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Departamento de Bioquímica.

# **Hipotiroidismo congénito central:** correlaciones clínico-genéticas e investigación de sus mecanismos moleculares

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Departamento de Bioquímica.
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# Hipotiroidismo congénito central: correlaciones clínico-genéticas e investigación de sus mecanismos moleculares

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CERTIFICA: Que Marta García González, Licenciada en Biología y Máster en Bioquímica, Biología Molecular y Biomedicina por la Universidad Complutense de Madrid, ha realizado bajo su dirección el trabajo de investigación titulado:

*Hipotiroidismo congénito central: correlaciones clínico-genéticas e investigación de sus mecanismos moleculares*

El que suscribe considera el trabajo realizado satisfactorio y apto para ser presentado como Tesis Doctoral en el Departamento de Bioquímica de la Facultad de Medicina de la Universidad Autónoma de Madrid.

Y para que conste donde proceda expiden el presente certificado en Madrid a 19 de Junio de 2017.

Fdo. José Carlos Moreno Navarro

# RESUMEN

*Marta García González. 2017.*

# *Hipotiroidismo congénito central: correlaciones clínico-genéticas e investigación de sus mecanismos moleculares*

# RESUMEN

El Hipotiroidismo Congénito Central (HCC) es un grupo heterogéneo de patologías causadas por la disminución de la síntesis, secreción o bioactividad de la hormona tirotropa (TSH), que no consigue estimular correctamente una glándula tiroidea normal. Esta patología no es detectada en el cribado neonatal de hipotiroidismo congénito por TSH realizado en España y en la mayoría de países europeos. Por este motivo suele ser una entidad infra-diagnosticada. En el presente estudio se ha investigado fenotípica y genéticamente la cohorte de pacientes con HCC más amplia conocida hasta la actualidad. Esta serie de pacientes es única por contener una gran proporción de casos con HCC aislado (68%). En la discriminación etiológica de la enfermedad mediante test de TRH se han identificado 5 tipos de respuestas: dos de carácter hipofisario (P1 yP2), dos hipotalámicas (H1 y H2) y un tipo de respuesta normal. En un paciente con respuesta hipotalámica tipo H2 al test de TRH se identificó una hipoplasia hipofisaria e hipotalámica poniendo de manifiesto un origen mixto de la enfermedad. Además, se identificó un paciente con un fenotipo central clínicamente demostrado de hipotiroidismo e hipermetabolismo hipotalámicos con respuesta tipo H1 en el test de TRH.

La base molecular del HCC es ampliamente desconocida. En el presente estudio se han identificado mutaciones en tres de los cuatros genes asociados a HCC en humanos: *IGSF1*, *TRHR* y *TBL1X*. Defectos en *IGSF1* causaron hipotiroidismo congénito central y un fenotipo no descrito hasta la fecha de sobreestimulación del eje gonadotropo (FSH) durante la minipubertad neonatal, causante del macroorquidismo observado en este paciente, como se ha demostrado en ensayos funcionales *in vitro.* De este modo, se ha desvelado el mecanismo de actuación de IGSF1 en células tirotropas y gonadotropas: IGSF1 estimula la expresión de TRHR, e indirectamente de síntesis y bioactividad de TSH, por regulación negativa de la vía TGFβ-Smad; y reduce la expresión de FSHB por regulación negativa de la vía Activina-Smad. Además, se ha demostrado la expresión de *IGSF1*en tiroides pero su función en la glándula aún está por investigar. Se ha identificado un defecto leve en el *TRHR* en una familia de etnia gitana cuyos portadores heterocigotos muestran por primera vez un fenotipo de Hipertirotropinemia de origen central. También se han descrito nuevas características fenotípicas asociadas a defectos en *TBL1X* por mutaciones severas: trastorno por déficit de atención e hiperactividad, macrocefalia y malformación de Arnold-Chiari I. Algunas de estas características fenotípicas también se han observado en pacientes con variantes en otros factores integrados en el complejo NCoR-SMRT del que *TBL1X* forma parte, como son NCOR1 y GPS2. Factores no asociados previamente con HCC en humanos, que abren una nueva línea de investigación para desentrañar su patogenia molecular en la enfermedad.

# ABSTRACT

Central Congenital Hypothyroidism (CCH) is a heterogeneous group of pathologies caused by decreased synthesis, secretion or bioactivity of the thyrotropin hormone (TSH), which fails to properly stimulate a normal thyroid gland. This pathology is not detected in the TSH-based neonatal screening program for congenital hypothyroidism performed in Spain as in most European countries. For this reason, it is usually an under-diagnosed entity. In the present study, the cohort of patients with broader central congenital hypothyroidism (CCH) known to date has been genetically and phenotypically investigated. This series of patients is unique because it contains a large proportion of cases with isolated central hypothyroidism (68%). In the aetiological discrimination of the disease by TRH test, 5 types of responses have been identified: two pituitary (P1 and P2), two hypothalamic (H1 and H2) and one normal response type. In a patient with H2 hypothalamic response to the TRH test, pituitary and hypothalamic hypoplasia were identified, revealing a mixed origin of the disease. Moreover, we identified a patient with clinically demonstrated hypothalamic hypothyroidism and hypermetabolism and with hypothalamic H1 type response.

The molecular basis of CCH is largely unknown. In the present study, mutations have been identified in three of the four genes associated with CCH in humans: *IGSF1*, *TRHR* and *TBL1X*. Defects in *IGSF1* caused CCH and a non-described phenotype to date of over-stimulation of the gonadotropic axis (FSH) during neonatal minipuberty, causing macroorchidism observed in this patient, as demonstrated in functional *in vitro* assays. Thus, the mechanism of action of *IGSF1* in thyrotrophic and gonadotropic cells has been unveiled: IGSF1 stimulates the expression of *TRHR*, and indirectly of TSH synthesis and bioactivity, by negative regulation of the TGFβ-Smad pathway, and reduces *FSHB* expression by down-regulation of the Activin-Smad pathway. In addition, the expression of *IGSF1* has been demonstrated in the thyroid but its function in the gland has yet to be investigated. A mild defect in *TRHR* has been identified in family of Roma origin, whose heterozygous carriers show, for the first time, a phenotype of hyperthyrotropinemia. Novel phenotypic characteristics associated with defects in *TBL1X* have also been described caused by severe mutations: attention deficit hyperactivity disorder, macrocephaly, and Arnold-Chiari type I malformation. Some of these phenotypic characteristics have also been observed in patients with variants in other integrated factors of the NCoR-SMRT complex such as NCOR1 and GPS2, of which TBL1X is part. These factors not previously associated with CCH in humans, open a new line of future research that will unravel its molecular implications in the disease.

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# ÍNDICE

# ABREVIATURAS

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# ABREVIATURAS

CGA: subunidad alfa de glicoproteínas

CRE: elemento de respuesta a cAMP

CREB: proteína de unión a elementos de respuesta a cAMP

CREM: modulador de elementos de respuesta a cAMP

DCHP: deficiencia combinada de hormonas hipofisarias

DIO1: desyodasa de yodotironina 1

DIO2: desyodasa de yodotironina 2

DIO3: desyodasa de yodotironina 3

DR: receptor de dopamina

FSH: hormona folículoestimulante

FSHB: subunidad beta de la hormona folículoestimulante

FSHR: receptor de hormona folículoestimulante

GH: hormona de crecimiento

HC: hipotiroidismo congénito

HCC: hipotiroidismo congénito central

HPT: eje hipotálamo-hipófisis-tiroides

L-T4: levotiroxina

LH: hormona luteinizante

MSHα: melanocortina

NA: núcleo arcuato

NB: neuromedina

NPV: núcleo paraventricular del hipotálamo

NPY: neuropéptido Y

NPYR: receptor de neuropéptido Y

PCSK1: proconvertasa 1

PCSK2: proconvertasa 2

POMC: propiomelanocortina

PPII: piroglutamilpeptidasa II

PRL: prolactina

RXR: receptor X de retinoide

SSTR: receptor de somatostatina

T3: triyodotironina

T4: tiroxina

T4L: tiroxina libre

T4T: tiroxina total

TBG: globina de unión a tiroxina

THRB: receptor beta de hormona tiroidea

TR: receptor de hormona tiroidea

TSH: tirotropina

TSHB: subunidad beta de tirotropina

TSHR: receptor de tirotropina

TRE: elementos de respuesta de receptores de hormona tiroidea

TRH: hormona liberadora de tirotropina

TRHR: receptor de hormona liberadora de tirotropina

# INTRODUCCIÓN

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# Introducción

El Hipotiroidismo Congénito Central (HCC) es un grupo heterogéneo de patologías causadas por la disminución de la síntesis, secreción o bioactividad de la hormona tirotropa o tirotropina (TSH), que no consigue estimular correctamente una glándula tiroidea completamente normal. Esta patología no es detectada en el cribado neonatal de hipotiroidismo congénito por TSH realizado en la mayoría de países europeos. Por este motivo suele ser una entidad infra-diagnosticada, que se revela mayoritariamente con una sintomatología clínica moderada. La base molecular del HCC es ampliamente desconocida. Tan solo se han identificado mutaciones en cuatro genes como causa de HCC en humanos: los clásicos de *TSHB* y *TRHR*, que codifican la subunidad Beta de la TSH y el Receptor de la hormona liberadora de tirotropina (TRH), respectivamente, el gen *IGSF1* (*Immunoglobulin Superfamily factor 1*) cuyos defectos se han asociado a hipotiroidismo hipofisario y macroorquidismo, y recientemente el gen *TBL1X* (transducing β-like protein 1, X-linked) asociado a hipotiroidismo central y pérdida auditiva.
Dada la complejidad fisiológica de la regulación central de la síntesis de hormonas tiroideas, es obvio que los defectos genéticos que conducen a HCC han de abarcar necesariamente una base genética más amplia, más genes que, por el momento, son desconocidos.

### *Cribado neonatal de hipotiroidismo congénito*

El HCC no es detectado mediante el cribado neonatal del hipotiroidismo congénito (HC) realizado en España y en la mayoría de países europeos, que detectan la elevación de los niveles de TSH como marcador "reflejo" del hipotiroidismo de origen tiroideo, el más frecuente (Schoenmakers N *et al.*, 2015). Cuando se implantó el cribado poblacional de enfermedades metabólicas e hipotiroidismo en Europa, a principios de los años 80, Holanda optó por detectar todos los tipos de HC utilizando un método basado en la determinación de tiroxina (T4) total en el papel de filtro (Verkerk PH *et al.*, 1993). En 1995, Holanda mejoró su cribado neonatal de HC añadiendo a la cuantificación de los niveles de T4 y TSH la de la globulina de unión a tiroxina (TBG), lo que permite calcular el ratio T4t/TBG, que ha resultado muy eficaz como sustituto de la determinación de T4 libre, difícil de detectar en papel de filtro. Con esta modificación, la eficacia de detección del HCC se ha triplicado, objetivándose que el HCC alcanza una prevalencia mucho mayor de lo que se estimaba (1:16.000 recién nacidos) (van Tijn DA *et al.*, 2005). Se ha reportado que, por métodos ELISA en sangre de papel de filtro para determinar T4 libre, la incidencia de HCC es de 1 en 31.000 neonatos (Adachi M *et al.*, 2012).

Estos programas sugieren que esta incidencia probablemente representa una subestimación que podría ser susceptible de modificación con una implementación de estas técnicas adicionales en países donde aún no se están aplicando (Lanting CI *et al.*, 2005).

Aún no hay estudios que comparen el desarrollo psicomotor de los niños con HCC que son diagnosticados tardíamente versus aquellos que son detectados y tratados tempranamente gracias a los mencionados programas de cribado neonatal. Sin embargo, muchos niños diagnosticados después de los 3 meses de edad presentaron retraso psicomotor e intelectual cuando fueron evaluados entre los 2 y los 8 años de edad, sugiriendo los importantes beneficios derivados de una detección neonatal de esta enfermedad (Dacou-Voutetakis C *et al.*, 1990; Bonomi M *et al.*, 2001; Baquedano MS *et al.*, 2010). De esta forma se podrían prevenir déficits mentales así como se podrían detectar de forma temprana fallos concomitantes de otros ejes hormonales hipofisarios que ocurren en un 78% de los casos de HCC y que presentan una alta morbilidad y mortalidad (van Tijn DA *et al.*, 2008A; Pfäffle R & Klammt J, 2011).

Finalmente, un diagnóstico temprano del HCC podría suponer un gran avance en el conocimiento del comportamiento infantil de la enfermedad y ayudaría a la identificación de nuevos genes asociados a defectos en el eje hipotálamo-hipófisis, como ha sido el caso del hallazgo de defectos en *IGSF1,* identificados en pacientes con hipotiroidismo central detectado en cribado neonatal basado en la determinación de T4 (Sun Y *et al.*, 2012; Tajima T *et al.*, 2013; Nakamura A *et al*, 2013; Joustra SD *et al.*, 2013; Joustra SD *et al.*, 2016A).

### *Control central de la función tiroidea: mecanismos hormonales y moleculares*

Las hormonas tiroideas se sintetizan en la glándula tiroides en forma mayoritaria de T4, pero también de tri-yodotironina (T3), la forma biológicamente activa. Su síntesis y secreción están finamente controladas y reguladas desde estructuras centrales, tanto hipotalámicas como hipofisarias (Chiamolera MI & Wondisford FE, 2009). Existen dos señales hormonales principales implicadas en el control del eje hipotálamo-hipófisis-tiroides (HPT): la TRH producida por neuronas del núcleo paraventricular del hipotálamo (NPV) y la TSH sintetizada en la células tirotropas de la hipófisis, que modula la síntesis y secreción de las hormonas tiroideas por el tiroides (Figura 1A) (Chiamolera MI & Wondisford FE, 2009). En los tejidos, un sistema de *desyodasas de yodotironinas* activan (DIO1, DIO2) o inactivan (DIO3) estas yodotironinas según las necesidades individuales y específicas de cada tejido, este sistema también participa activamente en la retroalimentación negativa el eje central hipotálamo-hipófisis (Dentice M & Salvatore D, 2011). Por ello, las células tirotropas de la hipófisis representa la zona de convergencia de las señales estimuladoras e inhibidoras, que modulan

en magnitud y biopotencia el estímulo de TSH que tiene que llegar a la glándula tiroidea. Adicionalmente al sistema de retroalimentación negativa ejercido por las hormonas, existen vías independientes de hormona tiroidea que son capaces de anular la regulación ejercida por T4 o T3, en respuesta a condiciones fisiológicas como el ayuno, la bajada de temperatura o los ritmos circadianos controlados por circuitos neuronales (Costa-e-Sousa RH & Hollenberg AN, 2012). Con todo ello, la homeostasis de hormonas tiroideas es el resultado de un sistema muy controlado donde una desregulación central del eje puede derivar en un hipotiroidismo de origen central por desconexión de los bucles de retroalimentación negativa o generar señales autónomas que perturben el equilibrio de la regulación central donde convergen todos los mecanismos mencionados anteriormente (Dietrich JW *et al.*, 2012).

## *Mecanismos hormonales que controlan la acción de la TSH*

### 1. Señales estimulatorias de la síntesis y acción de la TSH

#### *Sintesis y degradación de TRH*

La TRH u hormona liberadora de tirotropina es un tri-péptido modificado (*piro*Glu-His-Pro) que se produce en el NPV de la parte anterior del hipotálamo. Se sintetiza en forma de un pro-péptido precursor de 242 aminoácidos que es procesado de forma post-traduccional por enzimas endopeptidasas (proconvertasas 1 y 2: *PCSK1 y 2*) que liberan 5 moléculas maduras de TRH por cada molécula de propéptido (Paez Espinosa V *et al.*, 2007; Martín MG *et al.*, 2013). Las moléculas de TRH son secretadas en la eminencia media para llegar a la hipófisis donde estimulan la síntesis, liberación y bioactividad de la TSH a través de su unión a receptores específicos (TRHR) en la membrana plasmática de la célula tirotropa. Una vez secretada en la eminencia media, la TRH puede ser degradada por el enzima piroglutamil peptidasa II (PPII), lo que sugiere una regulación adicional de la cantidad final de moléculas de TRH que llegan a estimular la célula tirotropa hipofisaria (Sánchez E *et al.*, 2009).

#### *Pulsatilidad de la secreción de TSH y TRH*

Al igual que otras hormonas hipofisarias, la TSH se libera de forma pulsátil. Estos pulsos tienen lugar sobre un ritmo circadiano que conduce a una secreción máxima de TSH a medianoche que disminuye progresivamente hasta la tarde del día posterior. El mecanismo mediante el cual tiene lugar esta pulsatilidad en la secreción de tirotropina es en gran parte desconocido, aunque se sabe que es de origen fundamentalmente hipotalámico (Brabant G *et al.*, 1986; Brabant G *et al*., 1990).

A su vez, la TRH se secreta también de forma pulsátil en el hipotálamo (Covarrubias L *et al.*, 1994). La TRH parece modular exclusivamente la amplitud de los pulsos de TSH, pero no la

frecuencia de éstos (Samuels MH *et al.*, 1993), sugiriendo que puede haber un componente hipofisario activo. Se ha demostrado el perfil secretor pulsátil de la TSH en varios modelos animales, como en hipófisis experimentalmente desconectadas del hipotálamo que mantienen la pulsatilidad (Willoughby JO *et al.*, 1977; Roelfsema F *et al.*, 2008; Custro N *et al.*, 1994; Mantzoros CS *et al.*, 2001; Paz-Filho G *et al.*, 2009).

***Desensibilización de los receptores de TRH (TRHR) hipofisarios al TRH***

En la hipófisis, la TRH secretada desde el hipotálamo estimula su receptor específico (TRHR) localizado en la membrana plasmática de las células tirotropas. Tras la activación del receptor se produce su rápida desensibilización. El receptor es fosforilado en residuos Ser/Thr de su cola citoplasmática por la GPCR kinasa 2 (GRK2). Posteriormente las arrestinas se encargan de internalizan al receptor por endocitosis, impidiendo rápidamente su disponibilidad para ser estimulado por más TRH. Una vez que la TRH desaparece del medio, la fosfatasa 1 comienza a desfosforilar los receptores de TRH de los endosomas que serán transportados nuevamente a la membrana para iniciar de nuevo el ciclo (Hinkle PM *et al.*, 2012).

***TRH controla la biopotencia de TSH***

La señalización por el receptor de TRH no sólo induce la transcripción del gen *TSHB*. También influye directamente en las modificaciones post-traduccionales (esencialmente la glicosilación) que se llevan a cabo en la TSH y que le otorgan una mayor bioactividad. Existen distintas formas de TSH glicosilada según el tipo de glúcidos que se añadan a asparagina (Glicosilacion-N) y la conformación final de cadenas de hidratos de carbono que se produzcan (Persani L *et al.*, 1998). Las diferentes formas de glicosilacion de la TSH pueden contribuir a la homeostasis tiroidea y estar implicadas en patología: un aumento de TSH sialilada se traduciría en una menor bioactividad y por tanto en un hipotiroidismo central (Gyves PW *et al.*, 1990). Los distintos patrones de glicosilación de TSH podrían también explicar la falta de correlación entre los niveles de TSH y T4 libre en algunos pacientes con hipotiroidismo central.

## 2. Señales inhibitorias de la acción de la TSH

Las hormonas tiroideas son las principales ejecutoras de la regulación negativa del eje, actuando en hipotálamo (controlando la secreción de TRH) y en hipófisis (controlando la secreción de TSH). Esta regulación se ejerce a través de tres ”asas” de retroalimentación negativas que tienen lugar a nivel central: un asa *larga* (acción de T3, T4 en el hipotálamo), un asa *corta* (T3, T4 sobre células tirotropas hipofisarias) (Figura 1A) y se postula otra *ultracorta* (por acción de la propia TSH a nivel local), que tiene también lugar en la hipófisis (Figura 1B).

***Asa larga de retroalimentación negativa (Tiroides-Hipotálamo)***

La fisiología de TRH es gobernada en sentido negativo por las hormonas tiroideas (Figura 1A). Éstas hormonas llegan a las neuronas del hipotálamo (NPV) productoras de TRH atravesando la barrera hemato-encefálica por transportadores específicos (OATP1C1) y desde el líquido cefaloraquídeo a través de los tanicitos, células de origen glial localizadas en la pared ventrolateral del tercer ventrículo. Los tanicitos expresan tanto el transportador de T4 MCT8, como la desyodasa DIO2, que activa la T4 hacia T3 que, a su vez, pasará a las neuronas hipotalámicas y ejercerá los efectos transcripcionales negativos sobre el promotor de *TRH*. Estas neuronas *hipofisiotropas* también expresan DIO3 (que degrada la T3 a productos inactivos) cuya expresión es estimulada por T3, sugiriendo la existencia de un mecanismo de regulación local para compensar variaciones en la disponibilidad intracelular de T3 (Crantz FR & Larsen PR, 1980; Galton VA *et al.*, 2009; Lechan RM & Fekete C, 2007).

Por último, la presencia de T3 también estimula la expresión de la PPII en los tanicitos. Estas células interaccionan con las neuronas hipotalámicas del NPV y pueden por tanto degradar el exceso de TRH liberada en la eminencia media. Así, la T3 regula negativamente la secreción hipotalámica de TRH en sus aspectos de síntesis y de degradación (Sánchez E *et al.*, 2009; Costa-e-Sousa RH & Hollenberg AN, 2012).

***Asa corta de retroalimentación negativa (Tiroides-Hipófisis)***

Las hormonas tiroideas regulan negativamente la producción de la TSH en células tirotropas. La T4 y en menor medida la T3 llegan a la célula desde el tiroides por circulación sistémica, donde la T4 es desyodada a T3 por la DIO2, desyodasa predominante en hipófisis. La T3, implicada directamente en la regulación transcripcional negativa, alcanza el núcleo y actúa por unión al receptor nuclear de hormona tiroidea (THRB), que constituye el principal componente de la regulación transcripcional negativa del promotor de *TSHB*. La isoforma mayoritaria de *THRB* en hipófisis es *THRB2*, pero la isoforma *THRB1* está también presente de forma residual. El complejo T3-THRB2 interacciona con elementos de respuesta (TRE) localizados en el promotor de *TSHB* inhibiendo en último término la expresión del gen (Figura 1C) (Cheng SY *et al.*, 2010).

***Asa ultracorta de retroalimentación negativa (Hipófisis: células tirotropas y folículo-estrelladas)***

También se ha identificado en modelos animales un sistema de control de la secreción de TSH de carácter local que implica a las células folículo-estrelladas del lóbulo anterior de la hipófisis, que expresan el Receptor de TSH (TSHR). La TSH sería secretada al espacio extracelular de la

célula tirotropa e interaccionaría con los receptores de TSH localizados en las células folículo-estrelladas, lo que activaría la vía de señalización JAK/STAT5a que induce la expresión de TGFβ2, un factor paracrino que actúaría sobre las células tirotropas regulando negativamente la secreción de TSH por unión a su receptor (Brokken LJ *et al.*, 2005) (Figura 1B). Asimismo, este efecto paracrino y autoregulatorio de la propia TSH podría también estar implicado en la secreción pulsátil de la TSH, contrarrestando desde la hipófisis el aumento de la TSH durante los picos pulsátiles (Prummel MF *et al.*, 2004).

***Plasticidad de la célula hipofisaria y trans-diferenciación entre tipos celulares***

En modelos animales se ha identificado un nuevo efecto de la hormona tiroidea durante la embriogénesis de la hipófisis (Tonyushkina KN *et al.*, 2014). El exceso de T4 causa la muerte de las células tirotropas antes de que comience su función reguladora a través del sistema de retroalimentación negativa. Cuando se elimina este exceso de T4 la masa celular tirotropa puede recuperarse lentamente. Esta regulación ejercida por la T4 en el desarrollo de las tirotropas puede tener implicaciones importantes para la producción funcional de TSH y para su punto óptimo durante la vida del individuo. De hecho, se han descrito hipotiroidismos congénitos centrales en niños nacidos de madres con hipertiroidismo gestacional. La transitoriedad de este fenotipo y la recuperación del número de tirotropas cuando cesa el exceso de T4, ponen de manifiesto la gran plasticidad celular del tejido hipofisario, que podría trans-diferenciarse entre diferentes células hipofisarias si fuese necesario (Vidal S *et al.*, 2000; Radian S *et al.*, 2003).

***Dopamina y somatostatina en el hipotálamo***

La expresión de *TSHB* también está sujeta a regulación negativa por péptidos hipotalámicos. La somatostatina y la dopamina son dos neurotransmisores secretados por el hipotálamo que actúan en diferentes células de la hipófisis, cómo las tirotropas. Se unen a receptores específicos acoplados a proteínas G regulando negativamente la expresión de *TSHB* (Missale C *et al.*, 1998; Theodoropoulou M & Stalla GK, 2013). La secreción de la TSH es inhibida a través de los receptores SSTR2 y 5 de somatostatina. La estimulación de estos receptores conduce a la inactivación de la adenilato ciclasa y la fosfolipasa C, contrarrestando la vía TRH-TRHR y disminuyendo la expresión de *TSHB* (Missale C *et al.*, 1998; Theodoropoulou M & Stalla GK, 2013). Adicionalmente, el receptor DR2 de dopamina inhibe la expresión de *POU1F1,* reduciendo en último término la expresión de *TSHB* y *PRL* (Elsholtz HP *et al.*, 1991).

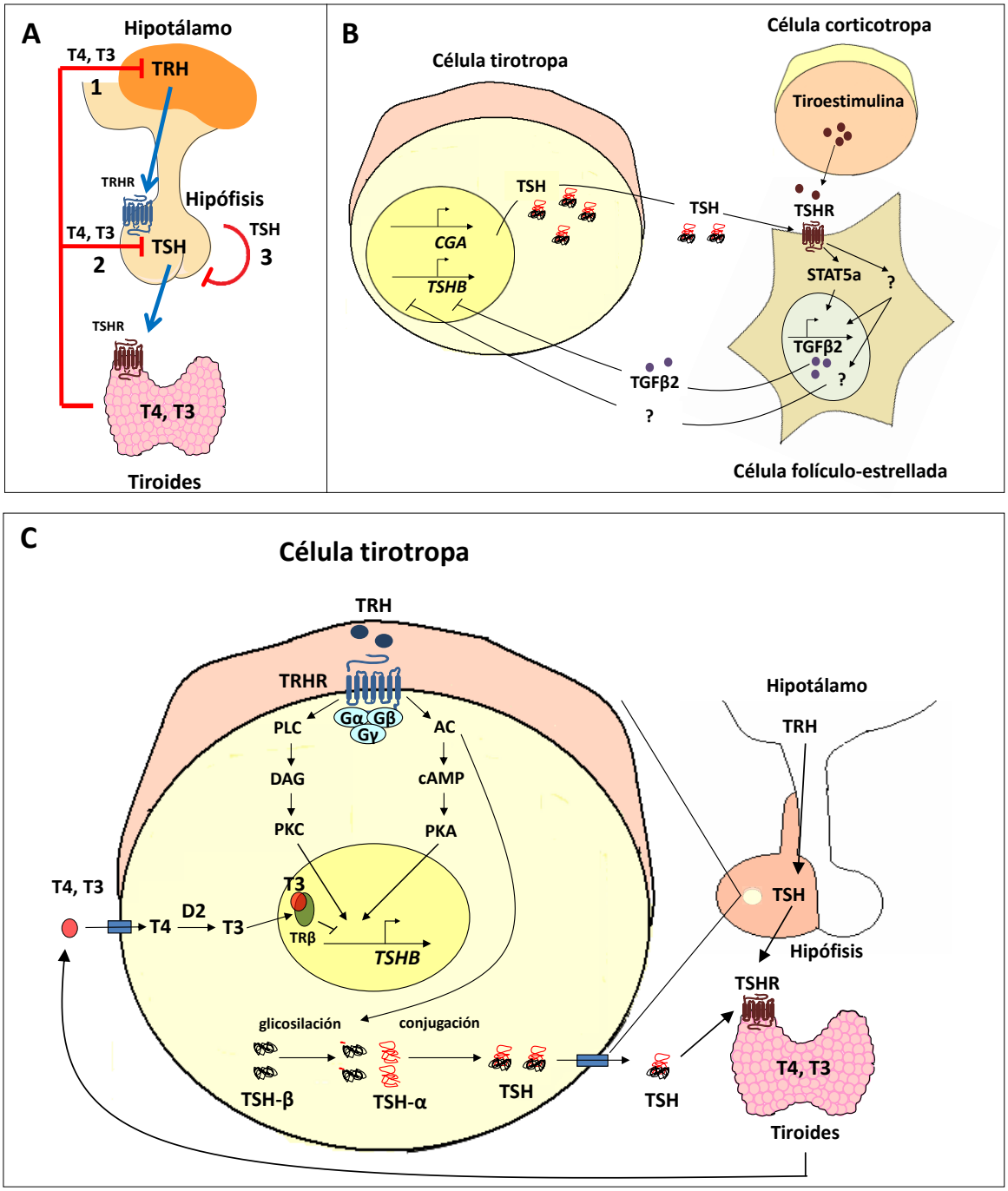



**Figura 1.** Regulación hormonal y transcripcional del eje tiroideo. (A) "Asas" de retroalimentación negativa del eje hormonal tiroideo: (1) larga hipotálamo-tiroides, (2) corta hipófisis-tiroides, (3) ultracorta dentro de la hipófisis. (B) "Asa" ultracorta de retroalimentación negativa por las células folículo-estrelladas de la hipófisis y las células corticotropas liberadoras de tiroestimulina. La secreción paracrina de la TSH en la hipófisis estimula los receptores de TSH de las células folículo-estrelladas que a su vez estimulan la vía de señalización de STAT5a, induciendo la secreción de TGFβ2 o moléculas similares que inhiben la síntesis de TSHB en las células tirotropas. La tiroestimulina también es capaz de estimular los receptores de TSH produciendo un efecto regulador paracrino. (C) Cascadas de señalización de síntesis, bioactividad y secreción de TSH desde el receptor de TRH en la célula tirotropa. La transcripción de *TSHB* es estimulada por dos vías de señalización diferentes (fosfolipasa C (PLC) y adenilato ciclasa (AC)) que son activadas por TRH. La vía adenilato ciclasa también participa en glicosilación y bioactividad de la TSH. La subunidad beta se conjuga con la alfa para formar el dímero activo de TSH que es capaz de estimular la glándula tiroidea. El receptor beta de hormona tiroidea es esencial en el "asa" corta de retroalimentación negativa hipófisis-tiroides, reprimiendo la síntesis de TSHB.

### 3. Otras señales involucradas en la señalización de TSH

***Tiroestimulina***

Se ha identificado un nuevo miembro de la familia de las hormonas glicoproteicas, la tiroestimulina, que podría participar en la homeostasis tiroidea pues es capaz de estimular potentemente el TSHR (Nakabayashi K *et al.*, 2002). Esta hormona es un dímero formado por las subunidades GPA2 y GPB5. El complejo A2/B5 es capaz de interaccionar con el TSHR y estimula más potentemente que la TSH la cascada de señalización por AMPc. La tiroestimulina se expresa tanto en hipófisis como en tiroides, ambos tejidos con presencia del TSHR (Nakabayashi K *et al.*, 2002; Prummel MF *et al.*, 2004; Brokken LJ *et al.*, 2005). Dentro de la hipofisis anterior se ha demostrado la co-expresión de ambas subunidades de la tiroestimulina en células corticotropas, que serían el origen de la secreción local de esta hormona (Okada SL *et al.*, 2006). Esto sugiere un efecto paracrino hipofisario que, a través de las células folículo-estrelladas, que expresan el TSHR, pudiera modular la secreción de la TSH. Aún no se ha demostrado la presencia de la tiroestimulina en suero, por lo que es improbable que ejerza una función endocrina clásica.

## *Mecanismos moleculares que controlan la acción de la TSH*

### 1. En el promotor del gen TRH.

***Control transcripcional positivo.***

La producción de TRH está controlada positivamente a nivel transcripcional por la presencia de elementos reguladores en el promotor del gen *TRH*, donde se unen factores como CREB (*cAMP response element binding protein*) y CREM (*cAMP response element modulator*) que activan la transcripción del gen (Chiappini F *et al.*, 2013) (Figura 2A). CREB aumenta en las células del NPV (productoras de TRH) a consecuencia del aumento de AMPc derivado de la estimulación del receptor de melanocortina (MSHα), denominado MC4R. MC4R es estimulado por la MSHα que es producida y secretada por células del vecino núcleo arcuato (NA) desde su precursor, la propiomelanocortina (POMC) (Sarkar S & Lechan RM, 2003). Otro estímulo fuerte para la transcripción de TRH es el frío, cuyo mediador molecular aún es desconocido (Perello M *et al.*, 2007).

***Control transcripcional negativo.***

La T3 a través de la unión a su receptor de hormona tiroidea beta (THRB2) ejerce un control negativo sobre la síntesis y liberación de la TRH en neuronas del NPV del hipotalámo. El complejo THRB2-T3 por interacción con elementos de respuesta (TRE) recluta una serie de factores nucleares co-represores o co-activadores como NCOA1 (SRC1: steroid receptor

coactivator) que participan en la represión de la expresión del gen *TRH* inducida por T3 (Guissouma H *et al.*, 2002; Takeuchi Y *et al.*, 2002) (Figura 2B). Sin embargo, las hormonas tiroideas no son el único control negativo del gen *TRH*: durante el ayuno, determinadas células hipotalámicas producen Neuropéptido Y (NPY) que estimula a su receptor (NPYR) en las células del NPV, lo que desencadena cascadas de inhibición de la transcripción del gen *TRH* (Sarkar S & Lechan RM, 2003). El NPY también regula negativamente la PCSK2 en neuronas productoras de TRH, disminuyendo la producción de pro-TRH (Cyr NE *et al.*, 2013).

**2. En el promotor del gen TSHB.**

***Control transcripcional positivo.***

En la hipófisis, la hormona liberadora de tirotropina (TRH), secretada desde el hipotálamo, estimula su receptor específico (TRHR) localizado en la membrana plasmática de las células tirotropas. La TRH unida a su receptor media la activación de la adenilato ciclasa, que conduce a un aumento de AMPc intracelular que, en último término, induce la transcripción del gen *TSHB* a través de CREB y CREB-binding protein (Figura 2C) (Hashimoto K *et al.*, 2000). Otra vía de señalización alternativa sería la activación de fosfolipasa C que produce la movilización de calcio de los compartimentos intracelulares al citoplasma, proceso que activa factores de transcripción que en último término favorecen la expresión génica (Marvin C & Gershengorn MD, 1997) (Figura 1C). Este complejo sistema de factores que estimulan la expresión de *TSHB* incluye POU1F1, GATA2, PITX1, PITX2, NR4A1, TRAP/SMCC mediator complex, TBL1X, NCOR1/SMRT, entre otras proteínas nucleares (Figura2D). Los factores funcionalmente más relevantes en la síntesis de TSHB son POU1F1 (Pit-1) y GATA2.

El factor **POU1F1** está formado por dos dominios funcionales: POU y el homeodominio de unión al ADN, ambos importantes para su función transcripcional sobre los promotores de las hormonas TSH, GH y PRL en tirotropas, somatotropas y lactotropas, respectivamente (Kashiwabara Y *et al.*, 2009; Ohba K *et al.*, 2011).

**GATA2** no sólo es fundamental en la síntesis de TSH sino también de las hormonas gonadotropas (FSH, LH). Ambos factores, GATA2 y POU1F1 interaccionan participando en la diferenciación de las células tirotropas de la hipófisis. En el promotor de *TSHB*, POU1F1 se une a tres sitios diferentes: P-RE2 y P-RE3 (localizados en -274/-278 y -402/-384, respectivamente) se encuentran cerca de elementos de repuesta a cAMP que contribuyen a la expresión de *TSHB* a través de la vía de señalización TRH-TRHR; P-RE1 es un elemento de respuesta poco definido (localizado entre los nucleótidos -128 y +8) que se encuentra cercano a elementos de respuesta de GATA2 protegiéndolos de la inhibición por la región supresora (Figura 2C) (Kashiwabara Y *et al.*, 2009; Ohba K *et al.*, 2011). La presencia de distintos elementos de

respuesta (P-REs) y su interacción funcional con elementos de respuesta GATA2, refleja la importancia de POU1F1 en la modulación transcripcional de *TSHB.*

**PITX1** y **PITX2** son dos factores de transcripción de funciones similares y complementarias, que se expresan de forma diferencial a lo largo del desarrollo y contribuyen a la formación de bolsa de Rathke (futura hipófisis), corazón, ojo, cavidad oronasal, dental y maxilares.

En el adulto, PITX2 es un factor necesario para el mantenimiento de la función tiroidea pues regula la expresión de otros factores (POU1F1, GATA2) necesarios para la transcripción de *TSHB* (Charles MA *et al.*, 2005; Castinetti F *et al.*, 2011). PITX1 está implicado en la respuesta de la hipófisis frente a un hipotiroidismo. Produce un aumento de la biosíntesis y secreción de la TSH cuando los niveles de T3 son bajos. Aunque para obtener una respuesta óptima es necesaria la presencia asociada de PITX2, PITX1 puede suplir las necesidades mínimas en el caso de que PITX2 estuviese alterado (Castinetti F *et al.*, 2011).

Otros factores implicados en la síntesis de tirotropina son NR4A1 (Nur77) y el complejo TRAP/SMCC (Thyroid hormone receptor-associated protein complex). **NR4A1** es un receptor nuclear huérfano que se expresa en células tirotropas, corticotropas y gonadotropas y su expresión está regulada positivamente por la cascada de señalización activada por TRH-TRHR. Estudios *in vivo* demuestran que NR4A1 activa la transcripción de *TSHB*, constituyendo un elemento regulador en el eje hipotálamo-hipófisis-tiroides (Figura 2D). Se cree que este factor actúa de forma cooperativa con POU1F1 y GATA2 en la transcripción de *TSHB* (Nakajima Y *et al.*, 2012).

El complejo **TRAP/SMCC**, compuesto por 30 subunidades altamente conservadas en el reino animal, forma parte de la maquinaria de regulación transcripcional celular, regulando positivamente la expresión de numerosos genes por interacción con receptores nucleares. En la hipófisis TRAP220 (MED1) favorece la síntesis de la TSHB, aunque el mecanismo a través del cual ejerce su efecto se desconoce (Fondell JD, 2013).

**NCOR1** junto con **TBL1X** juegan un papel fundamental en el control de la regulación génica mediada por hormona tiroidea. NCOR1 es un receptor nuclear co-represor que junto con otras proteínas co-represoras cómo TBL1X, forma parte del complejo represor de receptores de retinoides y hormona tiroidea (NCoR-SMRT). Este complejo interacciona con los receptores de retinoides y hormona tiroidea (TR-RXR) y en ausencia del ligando T3 favorece la activación basal de genes regulados negativamente por hormona tiroidea (*TRH* y *TSHB*) (Figura 2D) (Costa-e-Sousa RH & Hollenberg AN, 2012).

***Control transcripcional negativo***.

La T3 ejerce un control negativo en la síntesis y liberación de la TSH en células tirotropas por unión al receptor de hormona tiroidea (TR) y posterior reclutamiento de factores correpresores o coactivadores nucleares implicados en la regulación, como SRC1. En estudios in vivo se ha demostrado que NCoR1 por interacción con TR podría desempeñar un papel regulador "bi-direccional" (positivo y negativo) en la expresión de *TSHB*, determinando el punto óptimo de TSH de cada individuo (Astapova I *et al.*, 2011) (Figura 2E). Otro mecanismo transcriptional negativo que se ha sugerido como regulador de la transcripción de *TSHB* es el llevado a cabo por las vías de señalización de receptores de TGFB en tirotropas. Sin embargo, este mecanismo está poco investigado (Prummel MF *et al.*, 2004; Brokken LJ *et al.*, 2005).

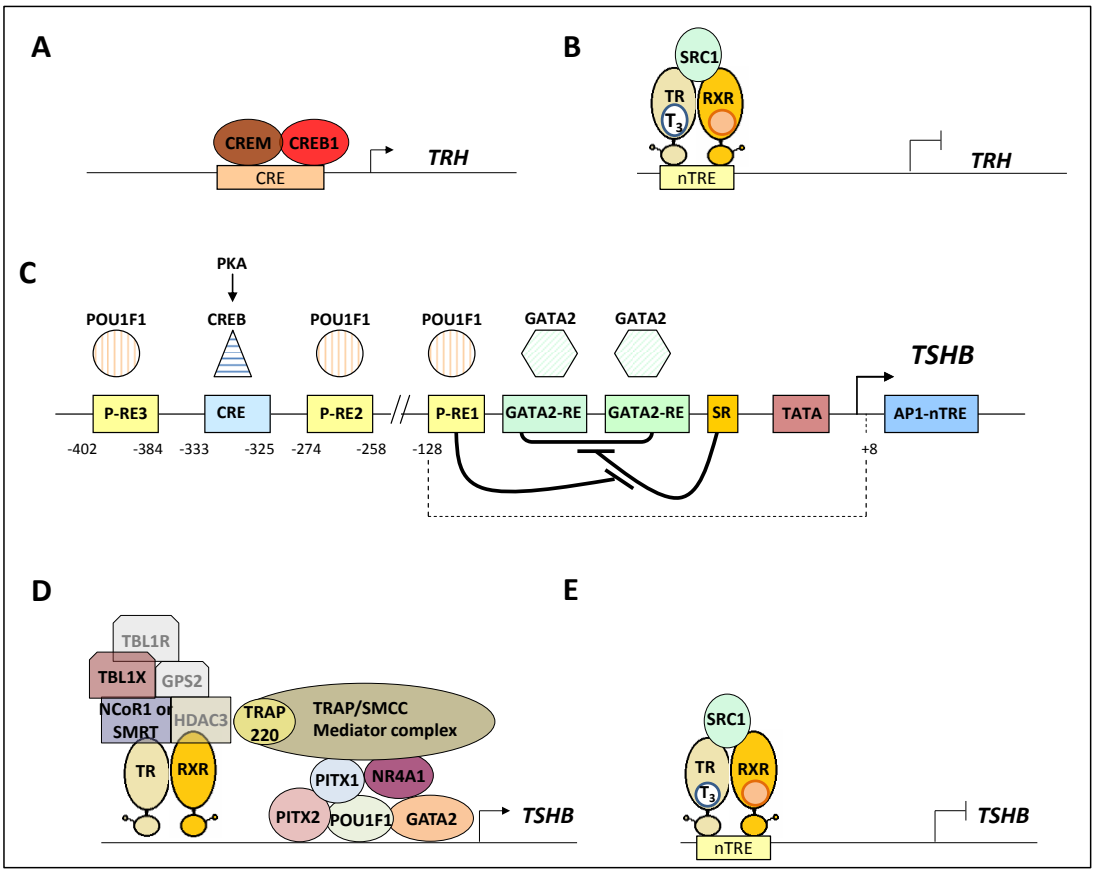



**Figura 2**. Factores de transcripción y proteínas nucleares implicados en desarrollo de hipófisis y expresión de los genes *TRH* y *TSHB*. (A) Control transcripcional positivo de *TRH*. La proteína de unión CREBP1 y el modulador CREM de elementos de respuesta por cAMP (CRE) se unen al promotor activando la transcripción de *TRH*. (B) Control transcripcional negativo de *TRH*. Los receptores de hormona tiroidea (TR) y retinoide (RXR) en presencia de T3 forman heterodímeros que interaccionan con elementos de respuesta negativos (nTRE) en el promotor, inhibiendo la expresión de *TRH*. Proteínas nucleares como SRC1 interaccionan con el complejo TR-T3-RXR favoreciendo esta inhibición. (C, D) Control transcripcional positivo de *TSHB*. (C) Esquema de la unión de los factores POU1F1 y GATA2 al promotor de *TSHB*. POU1F1 se une a los elementos de respuesta a POU1F1 1, 2 y 3 (P-RE1, P-RE2, P-RE3): P-RE2 y P-RE3 se localizan cerca de los elementos de respuesta a cAMP (CRE), mientras que P-RE1 se localiza cerca de los elementos de respuesta a GATA2 (GATA2-RE) evitando la inhibición de la acción de GATA2 por la región SR (región supresora). (D) El complejo mediador del silenciamiento de receptores de retinoides y hormona tiroidea (SMRT) formado por distintos factores como NCOR1 y TBL1X interaccionan con RXR y TR reprimiendo el efecto negativo de T3 sobre el promotor de *TSHB*. Los factores de transcripción POU1F1, GATA2, PITX1, PITX2, NR4A1 y el complejo TRAP/SMCC por unión al promotor de *TSHB* estimulan su expresión génica. (E) Control transcripcional negativo de *TSHB* ejercido por el complejo TR-RXR en presencia de ligando T3 y favorecido por receptores nucleares (SRC1).

### *Modelos animales de HCC*

La base genética del HCC es en gran parte desconocida en humanos. Por ello, se han generado distintos modelos animales que pueden ayudarnos a identificar en la clínica a pacientes con rasgos fenotípicos similares a los que presentan estos modelos, aportando conocimientos para establecer una mejor relación fenotipo-genotipo en esta patología humana (Tabla 1).

| HC | Modelo | Gen | Función | Fenotipo | R |
|---|---|---|---|---|---|
| **HC Hipotalámico** | Ratón KO $^{-/-}$ | *Trh* | Síntesis, bioactividad y liberación de TSH | ↑ sTSH con ↓ bioactividad, ↓ $sT_4$, hiperglicemia | 1 |
| | Ratón KO $^{-/-}$ | *Pcsk1*<br>*Pcsk2* | Endopeptidasas que genera los péptidos maduros de TRH a partir de la pro-TRH | ↓ péptidos activos de TRH,<br>↓ $sT_3$ (*Pcsk1* KO), N $sT_3$ (*Pcsk2* KO) | 2 |
| | Rata tratada con inhibidor de PPII | *PPII* | Peptidasa que degrada e inactiva la TRH | ↑ sTSH (estimulada por TRH y por frío) | 3 |
| | Ratón KO $^{-/-}$ en NPV* | *Creb1* | Factor de transcripción: regula positivamente la síntesis de TRH | N sTSH, N $sT_4$, N $sT_3$<br>↑ mRNA *Trh* compensatorio (por ↑ mRNA *Crem)* | 4 |
| | Ratón $^{Ob/Ob}$ | *Lep* | Regula la expresión de pro-TRH y Pcsk1 y Pcsk2 | Hipotiroidismo hipotalámico | 5 |
| **HC Hipofisario** | Ratón KO $^{-/-}$ | *Trhr1* | Media el efecto de la TRH en tirotropas | N sTSH, ↓ $sT_4$, ↓ $sT_3$, N sPRL | 6 |
| | Ratón KO $^{-/-}$ | *Igsf1* | Estimula la expresión de *TRHR* e inhibe la de *FSH* | ↓sTSH, N $sT_4$, ↓$sT_3$, ↓mRNA *Trhr*, ↓mRNA *Trh* | 7<br>8 |
| | Ratón KO $^{-/-}$ | *Cga*<br>*(Gsu)* | Subunidad alfa de las hormonas TSH, LH y FSH | Ausencia de sTSH, $sT_4$, pFSH y pLH<br>Hipogonadismo, infertilidad, enanismo | 9 |
| | Ratón KO $^{-/-}$ | *Gphb5* | Subunidad de la tiroestimulina, estimuladora del receptor de TSH | N sTSH, ↓ $sT_4$ (transitorio en ratón jóven)<br>Eutiroidismo en ratón adulto | 10 |
| | Ratón KO $^{-/-}$ | *Nmbr* | Receptor de neuromedina B.<br>Factor autocrino/paracrino de secreción de TSH | ↑ sTSH, N $T_4$, ↓$sT_3$<br>↑ mRNA *Trh,* ↑ mRNA *Trhr,* ↓ mRNA *Dio2* | 11<br>12 |
| | Ratón IS $^{snell/dwarf}$ | *Pou1f1*<br>*(Pit1)* | Factor de transcripción: regula positivamente la síntesis de TSH, PRL y GH | ↓ pTSH, ↓ pGH, ↓ pPRL<br>Enanismo | 13 |
| | Ratón KO $^{-/-}$ en hipófisis* | *Gata2* | Factor de transcripción: regula positivamente la síntesis de TSH, FSH y LH | ↓ sTSH , ↓ sFSH<br>conserva la fertilidad | 14 |
| | Ratón KI $^{Trap/Trap}$ | *Atbf1*<br>*(Zfhx3)* | Factor de transcripción: regula positivamente la síntesis de Pou1f1 | ausencia pTSHB, ↓ pGH, ↓ pPou1f1, N pPOMC<br>↓ mRNA *Pou1f1,* N mRNA *Lhx3*, N mRNA *Prop1* | 15 |
| | Ratón KO $^{-/-}$ | *Prop1* | Factor de transcripción implicado en diferenciación y organogénesis hipofisaria | ↓pTSH, ↓pGH, ↓pPRL, ↓ pFSH, ↓ pLH<br>Hipoplasia hipofisaria, hipogonadismo, enanismo | 16 |
| | Ratón KO $^{-/-}$ | *Lhx3* | Factor de transcripción implicado en organogénesis hipofisaria | Ausencia de pTSH, pGH, pLH, pPRL.<br>Ausencia mRNA *Pou1f1,* ↓mRNA *TPit.*<br>Hipoplasia hipofisaria. Letal | 17 |
| | Ratón KO $^{-/-}$ | *Lhx4* | Factor de transcripción implicado en organogénesis hipofisaria | ↓pTSH, ↓pGH, ↓pPRL, ↓ pLH, ↓ pPOMC<br>Hipoplasia hipofisaria. Letal | 18 |
| | Ratón KO $^{-/-}$ | *Pitx1* | Factor de transcripción implicado en organogénesis hipofisaria | ↓pTSH, ↓ pFSH, ↓ pLH<br>Anomalías en la línea media. Letal | 19 |
| | Ratón hipomórfico | *Pitx2* | Factor de transcripción implicado en organogénesis hipofisaria | ↓pTSH, ↓pGH, ausencia de pFSH y pLH, ↓ mRNA *Pou1f1,* ↓ mRNA *Gata2*. Letal al nacimiento. | 20 |
| | Ratón KO $^{+/-}$ | *Sox2* | Factor de transcripción implicado en desarrollo de hipófisis, SNC y placodas | ↓pTSH, ↓pGH, ↓pPRL, ↓ pLH<br>Subfertilidad masculina, hipogonadismo, enanismo | 21 |
| | Ratón KO $^{-/-}$ | *Sox3* | Factor de transcripción implicado en desarrollo de hipófisis y línea media del SNC | ↓pTSH, ↓pGH, ↓ pFSH, ↓ pLH<br>Hipogonadismo, enanismo, defectos hipotalámicos | 22 |
| | Ratón KO $^{-/-}$ | *Hesx1* | Factor de transcripción implicado en desarrollo de hipófisis, nervio óptico y cerebro | Displasia de bolsa de Rathke, cerebro y ojos.<br>Agenesia del cuerpo calloso y septum pellucidum | 23 |
| | Ratón KO $^{+/-}$ | *Trap220*<br>*(Med1)* | Co-activador transcripcional | ↓ mRNA *TshB*<br>Retraso en el crecimiento | 24 |
| | Ratón KI ΔID | *Ncor1* | Receptor nuclear co-represor que controla la regulación génica mediada por $T_3$ a través de TR | N sTSH, ↓ $sT_4$, ↓ $sT_3$<br>Crecimiento normal y tamaño tiroides normal | 25 |
| **Resistencias centrales a HT** | Ratón KO $^{-/-}$ | *Dio2* | Desyodasa 2 de $T_4$ a $T_3$ (activadora) | ↑ sTSH, ↑ $sT_4$, N $sT_3$ | 26 |
| | Ratón KO $^{-/-}$ | *Ncoa1*<br>*(Src1)* | Co-activador nuclear que controla la regulación génica mediada por $T_3$ a través de TR | ↑ sTSH, ↑ s $T_4$, ↑ $sT_3$ | 27 |
| | Ratón KO $^{-/-}$ | *Rxrg* | Receptor retinoide que interacciona con el TR | ↑ sTSH, ↑ s $T_4$, ↑ $sT_3$ | 28 |
| | Ratón KO $^{-/-}$ | *TRB* | Receptor de hormona tiroidea ($T_3$) β | ↑sTSH, ↑ $sT_4$, ↑ $sT_3$, bocio | 29 |

*(Leyenda en página siguiente)*

***Tabla 1***. *Modelos animales y celulares de hipotiroidismo central (HC) y alteraciones centrales de la regulación del eje tiroideo: genes manipulados genéticamente, función de la proteína codificada y fenotipos hormonales asociados*. KO: Knockout, KI: Knockin, IS: línea natural, -/-: inactivación homocigota, +/-: inactivación heterocigota, *: tejido específico, #: inactivación incompleta (el mRNA de la isoforma 4 de *Igsf1* se expresa), N: normal, ↑: elevada, ↓: disminuida, s: niveles en suero, p: niveles de proteína en tejido, SNC: sistema nervioso central, TR: receptor de hormona tiroidea, PRL: prolactina, FSH: hormona folículo estimulante, LH: hormona luteinizante, POMC: pro-opiomelanocortina, *Trh:* hormona liberadora de tirotropina, *Pcsk*: proconvertasa, *PPII:* piroglutamil peptidasa II; *Creb1:* proteína de unión a elementos de respuesta a cAMP, *Crem:* modulador de elementos de respuesta a cAMP, *Lep:* leptina, *Trhr1*: receptor 1 de TRH, *Igsf1:* factor 1 de la superfamilia de inmunoglobulinas, *Cga/Gsu:* subunidad alfa de hormonas glicoproteicas, *Gphb5:* subunidad beta 5 de hormona glicoproteica, *Nmbr*: receptor de neuromedina B, *Pou1f1/Pit1:* factor de transcripción 1 específico de hipófisis, *Gata2:* proteína 2 de unión a Gata, *Atbf1/Zfhx3*: factor de transcripción 1 de unión a AT, *Prop1:* proteína homeobox profeta de *Pit1*, *Lhx3* and *Lhx4:* proteínas LIM/homeobox, *Pitx1* and *Pitx2:* proteínas homeobox 1 y 2 de hipófisis, *Sox2* and *Sox3:* proteínas SRY-box 2 y 3, *Hesx1*: HESX homeobox 1, *Trap220/Med1:* subunidad 1 del complejo mediador, *Ncor1*: receptor nuclear co-represor 1, *Dio2*: desyodasa 2, *Ncoa1/Src1*: receptor nuclear co-activador 1, *Rxrg*: receptor retinoide gamma, *TRB:* receptor beta de $T_3$. R: References, 1: Yamada M et al., 1997, 2: Cyr NE et al., 2012, 3: Sánchez E et al., 2009, 4: Chiappini F et al., 2013, 5: van der Kroon PH et al., 1982, 6: Rabeler R et al., 2004, 7: Sun Y et al., 2012, 8: Bernard DJ et al., 2003, 9: Kendall SK et al., 1995, 10: van Zeijl CJ et al., 2010, 11: Oliveira KJ et al., 2006, 12: Oliveira KJ et al., 2014, 13: Camper SA et al., 1990, 14: Charles MA et al., 2006, 15: Qi Y et al., 2008, 16: Nasonkin IO et al., 2004, 17: Sheng HZ et al., 1996, 18: Sheng HZ et al., 1997, 19: Szeto DP et al., 1999, 20: Suh H et al., 2002, 21: Kelberman D et al., 2006, 22: Rizzoti K et al., 2004, 23: Dattani MT et al., 1998, 24: Ito M et al., 2000, 25: Astapova I et al., 2011, 26: Schneider MJ et al., 2001, 27: Weiss RE et al., 1999, 28: Brown NS et al., 2000, 29: Brent GA, 2000.

## *Hipotiroidismo central en humanos*

### *1. Diagnóstico e investigación etiológica del HCC*

El diagnóstico del hipotiroidismo congénito central requiere un alto grado de sospecha clínica (Yamada M & Mori M, 2008; Persani L, 2012). Los pacientes pueden presentar una disminución leve de T4 con una TSH baja, normal o levemente elevada en función de si el hipotiroidismo es predominantemente hipofisario o hipotalámico, respectivamente (Rose SR , 1995). Por tanto, la interpretación hormonal en algunas ocasiones es complicada y puede ser de gran ayuda el uso de otras herramientas como la relación TSH-T4 representada en el modelo de Dietrich *et al.,* que consiste en la representación gráfica de la homeostasis tiroidea a través de las respuestas normales del tiroides y la hipófisis en su sistema de regulación óptimo (Dietrich JW, 2012).

Otra herramienta útil en la determinación etiológica del hipotiroidismo central es el test de TRH, que se comenzó a utilizar a partir de los años 70 para diagnóstico de hipotiroidismo central e hipotiroidismo subclínico, debido a la baja sensibilidad de las determinaciones hormonales en esa década. A partir de los años 90 los ensayos hormonales de 2ª y 3ª generación hacen innecesario el test de TRH para el diagnóstico de los hipotiroidismos subclínicos y centrales, pero sigue se sigue usando en la diferenciación entre HCC secundario y terciario (Faglia G, 1998). El uso del test no ha estado exento de controversia y algunos autores han cuestionado su utilidad en el diagnóstico y clasificación de pacientes con defectos centrales, argumentando que, la existencia de solapamientos en la respuesta de TSH entre pacientes con defectos hipotalámicos e hipofisarios hace difícil su discriminación (Dayan CM, 2001; Mehta A *et al.*, 2003). Para maximizar la eficacia diagnóstica del test, otros autores

realizaron un estudio detallado de la dinámica de subida y de baja de la TSH en los test de diferentes pacientes durante 180 minutos de duración. Este test está basado en la determinación de la TSH a distintos tiempos y en el cálculo de ratios que permitan la valoración de la dinámica y magnitud de la respuesta (15'/0', 30'/0') y la recuperación de la TSH basal (180'/0') (Van Tijn DA *et al.*, 2008B). El hipotiroidismo hipotalámico se valora por criterios cualitativos, se produce un aumento de la magnitud de la respuesta que puede retrasarse en el tiempo y tener una ausencia de recuperación de la TSH basal a las 3 horas. En el hipotiroidismo hipofisario se valoran criterios fundamentalmente cuantitativos, presentando una respuesta disminuida de TSH a la TRH con recuperación completa de la TSH basal a los 180 minutos (Van Tijn DA *et al.*, 2008B).

El estudio por resonancia magnética nuclear cerebral de la morfología de la hipófisis y el hipotálamo en pacientes con sospecha de HCC constituye otra herramienta diagnóstica en la determinación del origen de la enfermedad.

Por último, para completar la batería de herramientas útiles en el diagnóstico del HCC, la investigación de la bioactividad de TSH en los pacientes puede ayudar a esclarecer el estado del eje hipotálamo-hipófisis-tiroides en estos individuos. La determinación de la biopotencia de la TSH se puede llevar a cabo de forma indirecta mediante la medida de los niveles de T4 y T3 antes y después de estímulo con TRH en el test (Yamada M & Mori M, 2008; Horimoto M *et al.*, 1995), o directamente en el suero de los pacientes, recogido al principio y al final del test de TRH. Este suero se pone en contacto con un sistema celular *in vitro* que contiene elementos de respuesta a TSH bioactiva, cuantificando de esta manera la potencia que presenta la propia TSH de los pacientes.

El correcto diagnóstico de niños y adultos es muy importante para implementar un tratamiento que corrija el defecto (Beck-Peccoz P, 2011). Sin embargo, las mujeres embarazadas requieren especial atención, puesto que podrían desarrollar una hipotiroxinemia gestacional que puede pasar desapercibida en un diagnóstico de rutina por evaluación de los niveles de TSH. La disminución de T4 en la madre puede agravar el estado de hipotiroidismo del feto, quien también puede ser portador del defecto genético, resultando en una privación cerebral de hormona tiroidea que puede interferir en el desarrollo normal del cerebro fetal (Pine-Twaddell E *et al.*, 2013).

## 2. *Formas genéticas de Hipotiroidismo Central en humanos*

### *Deficiencia aislada de TSH*

Defectos en *TRHR*

Tan solo se conocen 3 casos familiares no relacionados con mutaciones en el receptor de TRH (*TRHR*), de herencia autosómica recesiva (Collu R *et al.*, 1997; Bonomi M *et al.*, 2009; Koulouri O *et al.*, 2016) (Figura 3A). El primer paciente descrito presentaba una heterocigosis compuesta de 2 mutaciones: un codón de parada prematuro p.R17X en un alelo, y en el otro, una deleción de 3 aminoácidos p.S115_T117del asociada a otra mutación de cambio de aminoácido, p.A118T (Collu R *et al.*, 1997). La misma mutación p. R17X fue identificada en homocigosis en el segundo paciente (Bonomi M *et al.*, 2009), mientras que el tercer paciente tenía una mutación de cambio de aminoácido p.P81R (Koulouri O *et al.*, 2016). Todas estas variantes patogénicas afectan de forma severa a la señalización del receptor (Collu R *et al.*, 1997; Bonomi M *et al.*, 2009; Koulouri O *et al.*, 2016).

Ninguno de los tres pacientes fue diagnosticado en el cribado neonatal utilizado en su país de origen basado en los niveles de TSH. Los dos primeros fueron detectados a las edades de 9 y 11 años, respectivamente, por presentar talla baja y síntomas compatibles con hipotiroidismo (fatiga, bajo rendimiento escolar) (Collu R *et al.*, 1997; Bonomi M *et al.*, 2009), mientras que el tercero fue diagnosticado y tratado a los dos meses de edad (Koulouri O *et al.*, 2016). En todos los casos, el perfil hormonal tiroideo mostró una TSH normal (con sospecha de bioactividad baja) y una T4 moderadamente baja. Todos los portadores heterocigotos eran eutiroideos. En los dos primeros casos se realizó el test de TRH que mostró una respuesta plana de la TSH y de la prolactina en los probandos (Collu R *et al.*, 1997; Bonomi M *et al.*, 2009).

La talla baja de los dos primeros pacientes puede ser atribuida a la presencia de hipotiroidismo en la infancia, ya que en el primer paciente se ha visto un incremento de su crecimiento después del tratamiento con levotiroxina y una talla final del segundo paciente acorde con su talla diana parental (Collu R *et al.*, 1997; Bonomi M *et al.*, 2009). Ninguno de los pacientes de diagnóstico tardío presentó déficits cognitivos o psicomotores evidentes lo que contrasta con el severo cretinismo observado en pacientes con mutaciones en *TSHB*, sugiriendo una mayor levedad del hipotiroidismo infantil por defectos en *TRHR* (Hayashizaki Y *et al.*, 1989).

En todos ellos, la levedad de los fenotipos apoya el alto grado de casos que pueden quedar sin diagnosticar o recibir un diagnóstico tardío.

Otros fenotipos pueden derivarse de una incorrecta señalización TRH-TRHR pero hasta la fecha no se han identificado defectos en humanos en *TRH*. Sin embargo, el ratón deficiente en TRH muestra hipotiroidismo hipotalámico con elevación leve de TSH con bioactividad reducida (Yamada M *et al.*, 1997).

Defectos en *TSHB*

Al igual que otros miembros de la familia de hormonas glicoproteicas, la TSH es una proteína *nudo-cistina* dimérica compuesta por una cadena alfa y una cadena beta. En el gen de *TSHB* se han descrito 12 mutaciones diferentes, que conducen a una deficiencia aislada de TSH de herencia autosómica recesiva (Figura 3B) (Hayashizaki Y *et al.*, 1989; Dacou-Voutetakis C *et al.*, 1990; Hermanns P *et al.*, 2014; Nicholas AK *et al.*, 2017). Como el cribado neonatal realizado en la mayoría de los países se basa en la detección de incrementos de TSH, los pacientes con defectos en el gen *TSHB* no se detectan y su diagnóstico y tratamiento es tardío. Muchos de ellos son detectados clínicamente después de los 3 meses de edad (3-8 meses) con retraso mental y neuromotor en evaluaciones psicométricas a los 2-8 años de edad (Dacou-Voutetakis C *et al.*, 1990; Bonomi M *et al.*, 2001; Baquedano MS *et al.*, 2010). Por otro lado, los pacientes que son excepcionalmente diagnosticados y tratados en las primeras semanas de vida, con síntomas de hipotiroidismo, evitan el desarrollo de retraso mental (Brumm H *et al.*, 2002). En todos los casos la respuesta de TSH en el test de TRH es muy baja o prácticamente nula, mientras que la respuesta de prolactina es completamente normal.

La mutación más frecuente en este gen es una deleción de 1 nucleótido en el codon 125 (c.T373del,p.C125Vfs134X) que lleva a una proteína truncada, no detectada en inmunoensayos de TSH y es biológicamente inactiva sobre el TSHR, demostrado por ensayos *in vitro* (Medeiros-Neto G *et al.*, 1996).

Otra mutación frecuente y deletérea del gen *TSHB* fue identificada en un paciente con deficiencia aislada de TSH y cretinismo provocado por la sustitución de un aminoácido, p.G49R, localizado en la región CAGYC de la proteína (Hayashizaki Y *et al.*, 1989) (Figura 3B). El análisis tridimensional del dímero de TSH revela que la región CAGYC es importante en la heterodimerización de las cadenas alfa y beta de la TSH, que constituyen la molécula activa. Los pacientes con esta mutación tienen una TSH indetectable en suero y una disminución severa de T4 y T3 (Hayashizaki Y *et al.*, 1989).

Un fenotipo clínico característico completo de mutaciones en *TSHB* fue identificado en una niña de 75 días de edad referida por síntomas severos de hipotiroidismo, que además presentó una hipófisis hiperplásica y un tiroides hipoplásico (Bonomi M *et al.*, 2001). Este paciente presentaba la mutación p.Q69X en *TSHB*, que deleciona el 60% de la secuencia carboxi-terminal de la proteína y genera una pérdida completa de bioactividad (Figura 3B) (Bonomi M *et al.*, 2001). Recientemente, se ha descrito la deleción completa del gen *TSHB* en un paciente que a los 45 días de vida presentó fenotipo severo de hipotiroidismo, con niveles indetectables de TSH y T4 y T3 bajas (Hermanns P *et al.*, 2014) (Figura 3B).

Se han identificado 8 mutaciones adicionales en *TSHB* hasta la fecha: mutación de codón de parada (p.E32X), de pérdida del marco de lectura (p.F77Sfs82X), de splicing (c.162+1G>A, c.162+5 G>A) y de cambio de aminoácido (p.C105R, p.C108Y, p.E32K, p.Met1?) (Nogueira CR *et al.*, 1999; Pohlenz J *et al.*, 2002; Sertedaki A *et al.*, 2002; Morales AE *et al.*, 2004; Nicholas AK *et al.*, 2017; Özhan B *et al.*, 2017).

Defectos en *IGSF1*

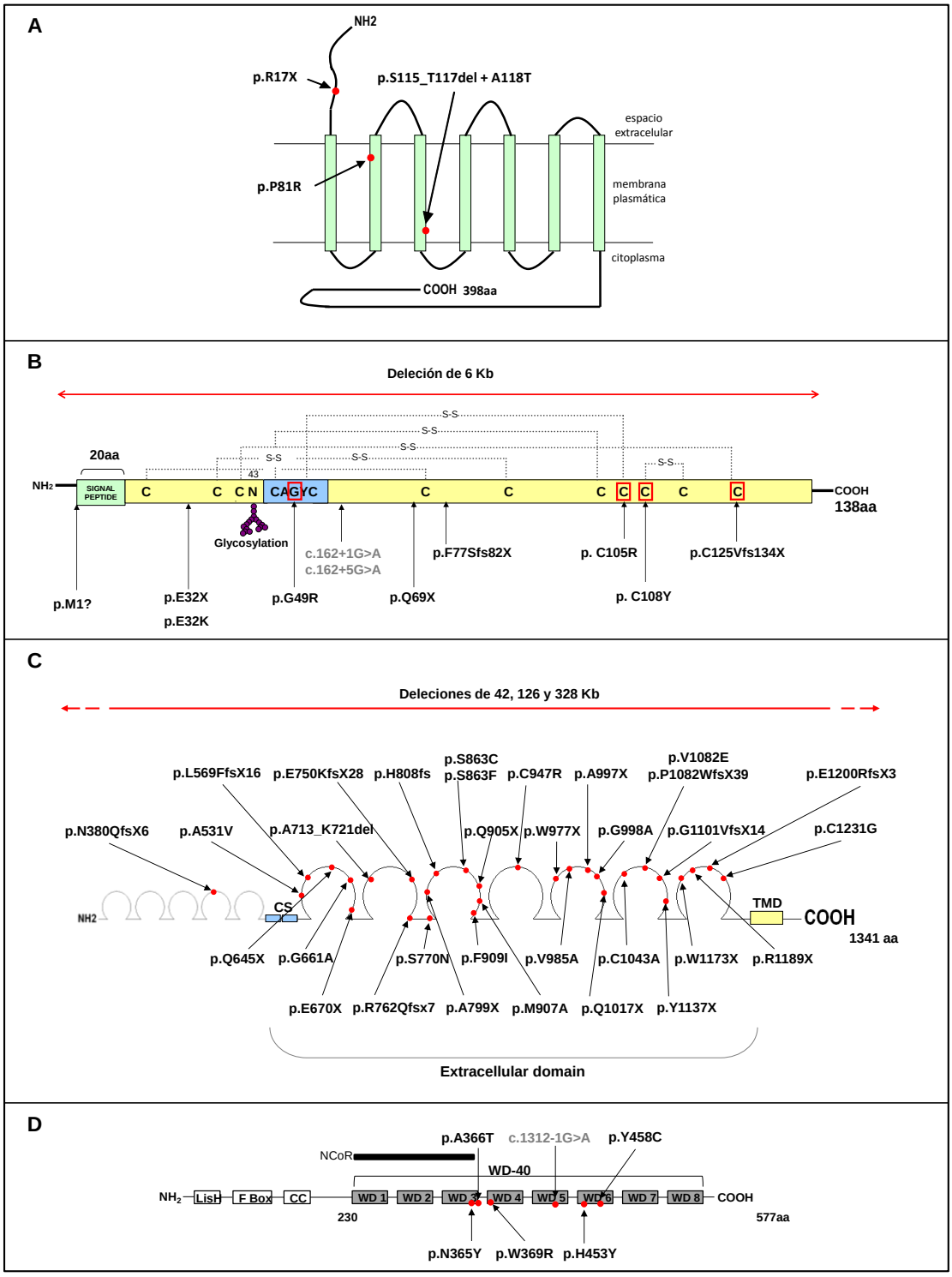
*IGSF1* es un gen codificado en el cromosoma X que se ha asociado recientemente a hipotiroidismo central y macroorquidismo pero que en la actualidad constituye la causa genética más frecuente de HCC (Joustra SD *et al.*, 2014; Joustra SD *et al.*, 2016A). Los defectos en este gen son de herencia ligada al X, por tanto se han identificado mayormente en varones, aunque también se han descrito mujeres con fenotipo leve de HCC (Joustra SD *et al.*, 2014). El macroorquidismo es un rasgo fenotípico variable en pacientes con estos defectos y su patogénesis es desconocida. La asociación entre hipotiroidismo y macroorquidismo se ha descrito clásicamente en la era pre-cribado de hipotiroidismo congénito, en niños tardíamente diagnosticados de hipotiroidismo (Castro-Magaña M *et al.*, 1988). El incremento de tamaño testicular en estos casos era debido a una estimulación cruzada del receptor de FSH testicular por una TSH elevada de forma crónica en estos niños (Anasti JN *et al.*, 1995). Sin embargo, los defectos en *IGSF1* muestran una deficiencia de TSH, por lo que necesariamente el mecanismo patogénico del macroorquidismo en estos casos no puede ser atribuido a lo clásicamente descrito y se requieren futuras investigaciones para determinar la relación de esta característica fenotípica con el defecto en *IGSF1*.

Los varones con alteraciones en *IGSF1* pueden presentar otros defectos hipofisarios hormonales adicionales como una deficiencia parcial de prolactina o GH (Sun Y *et al.*, 2012; Tajima T *et al.*, 2013; Nakamura A *et al.*, 2013; Joustra SD *et al.*, 2013; Joustra SD *et al.*, 2016A). También se ha descrito hipoplasia tiroidea, pubertad retrasada, sobrepeso y déficit de atención en estos pacientes. Por lo que la deficiencia en *IGSF1* constituye una entidad fenotípicamente heterogénea de defectos con una manifestación común, el hipotiroidismo congénito central.

Se han descrito más de 30 familias con mutaciones diferentes en *IGSF1*, la mayoría identificadas países que utilizan el cribado neonatal basado en T4.

Esta enfermedad está caracterizada por hipotiroidismo central de origen hipofisario, mostrando una reducción de la respuesta de TSH en el test de estímulo con TRH. Algunos pacientes presentaron hipoplasia tiroidea (Joustra SD *et al.*, 2016A). Muchos pacientes desarrollan un macroorquidismo temprano y progresivo y en ocasiones una pubertad

retrasada que requiere tratamiento con testosterona en algunos casos. Además, se ha demostrado que la secreción pulsátil de FSH en estos pacientes está aumentada un 200% (Joustra SD, 2016B).




*(Leyenda en página siguiente)*

**Figura 3.** Mutaciones identificadas en proteínas asociadas a hipotiroidismo congénito central. (A) En TRHR sólo se han identificado 3 mutaciones: una en el dominio extracelular, dos en el segundo y tercer dominio transmembrana, respectivamente. (B) La TSHB contiene un péptido señal de 20 aminoácidos, un dominio CAGYC muy conservado de heterodimerización entre las subunidades alfa y beta y una arginina 43 que es glicosilada, modulando la bioactividad de la TSH. Se han identificado 12 mutaciones en esta proteína, siendo la más frecuente la p.C125Vfs134X. Estas mutaciones están nombradas según las guías actuales (den Dunnen JT *et al.*, 2016), incluyendo los 20 aminoácidos del péptido señal (por tanto, puede haber diferencias en la nomenclatura de los mutantes publicados en artículos antiguos). (C) IGSF1 presenta 12 dominios inmunoglobulina, un domino transmembrana y una cola intracelular. La proteína se corta por el conector hidrofóbico entre los dominios 5 y 6. Se han identificado 32 mutaciones en la parte extracelular de la proteína. (D) TBL1X se ha asociado recientemente a hipotiroidismo central y sólo se han identificado 6 mutaciones, todas en los dominios repetidos WD-40 de interacción proteica.

IGSF1 es una proteína de membrana con 12 dominios inmunoglobulina muy similar a un receptor quinasa (Figura 3C) (Mazzarella R *et al.*, 1998). Se han identificado 31 mutaciones que alteran los dominios extracelulares de la proteína: 13 generan un cambio de aminoácido, 10 generan un codón de parada y 8 mutaciones de cambio del patrón de lectura por deleciones, inserciones o duplicaciones. También se han identificado tres deleciones completas del gen (Asakura Y *et al.*, 2015; Nishigaki S *et al.*, 2016; Tenenbaum-Rakover Y *et al.*, 2016; Hughes JN *et al.*, 2016) (Figura 3C). Sin embargo, no se ha establecido una relación clara genotipo-fenotipo entre el tipo de mutación o su localización celular y la severidad o inicio del hipotiroidismo.

Defectos en *TBL1X*

*TBL1X* es un gen cuyas mutaciones se han identificado recientemente como causa genética de hipotiroidismo central, está codificado en el cromosoma X y su proteína es una subunidad esencial del complejo represor NCoR-SMRT del receptor de T3 (Yoon HG *et al.*, 2003; Astapova I *et al.*, 2011). En promotores regulados de forma negativa (*TRH* y *TSHB*), NCoR-SMRT promueve la activación basal del promotor en ausencia de T3, regulando eje hipotálamo-hipófisis-tiroides (Astapova I *et al.*, 2011; Costa-e-Sousa RH & Hollenberg AN, 2012).

En un principio los defectos en *TBL1X* se habían asociado únicamente con pérdida auditiva, pero recientemente se han descrito pacientes con hipotiroidismo central (Bassi MT *et al.*, 1999; Heinen CA *et al.*, 2016).

Esta enfermedad está ligada al X, por tanto se expresa mayoritariamente en varones, aunque también se han identificado mujeres con hipotiroidismo central (Heinen CA *et al.*, 2016). Se han descrito un total de 6 familias con defectos en este gen, dentro de las cuales no todos los individuos portadores de mutaciones (la mayoría mujeres) presentaron hipotiroidismo. El hipotiroidismo causado por mutaciones en este gen se manifiesta con TSH normal y T4 baja y algunos presentaron síntomas variables de fatiga, obesidad o sobrepeso (Heinen CA *et al.*, 2016). Todos los individuos portadores de mutaciones en los que se realizó el test de TRH, mostraron una respuesta de TSH normal al estímulo con TRH. Ningún paciente presentó

alteraciones en la hipófisis aunque todos los individuos con mutación y HCC tenían una glándula tiroidea de tamaño disminuido. Una alteración extra-tiroidea asociada en estos pacientes y que ya había sido previamente descrita en personas con defectos en *TBL1X* es la pérdida auditiva a altas frecuencias en estudio por audiometría (Heinen CA *et al.*, 2016).

TBL1X es una proteína con dominios repetidos WD40 que median la interacción con otras proteínas, por lo que los miembros de esta familia tienen funciones reguladoras (Yoon HG *et al.*, 2003). Las mutaciones identificadas en *TBL1X* se hallan en estos dominios de interacción WD40. Se han identificado 6 mutaciones en 19 individuos pertenecientes a 6 familias: 5 mutaciones son de cambio de aminoácido y una de splicing. Estudios estructurales de proteínas sugieren que estas mutaciones alteran la estructura y función de la proteína (Figura 3D) (Heinen CA *et al.*, 2016).

***Deficiencia de TSH en el contexto de deficiencias combinadas de hormonas hipofisarias***

El desarrollo embriológico de la glándula hipofisaria es muy complejo, pues consiste en la generación final de 6 líneas celulares distintas a través de la expresión en cascada de distintos factores de transcripción, o proteínas morfogenéticas de hueso, incluyendo otros específicos de cada una de los tipos celulares pituitarios. Estos factores hipofisarios se expresan de forma coordinada en el tiempo y el espacio (Davis SW *et al.*, 2010; Wang Y *et al.*, 2010; de Moraes DC *et al.*, 2012). Hay factores de expresión temprana como HESX1, LHX3, LHX4, PITX1, PITX2, SOX2 o SOX3, que intervienen en la formación inicial de la hipófisis. No obstante, el desarrollo de los diversos linajes de células hipofisarias requiere la actividad de factores que se expresan más tardíamente y que intervienen en la diferenciación final de las células somatotropas, lactotropas, tirotropas, gonadotropas, corticotropas y melanotropas (Davis SW *et al.*, 2010).

La complejidad de los mecanismos moleculares que regulan el eje hormonal tiroideo en hipotálamo e hipófisis anticipa la diversidad de defectos genéticos que han de ser la causa de hipotiroidismo central, una diversidad que en el momento solamente se vislumbra. Es el HCC una patología que necesita de un alto índice de sospecha en las consultas clínicas, teniendo en cuenta el posible agrupamiento familiar de hipotiroidismos leves, que habrá que diferenciar del hipotiroidismo leve primario. Ante sospecha de HCC debemos caracterizar en detalle clínicamente a los pacientes, sirviéndonos de los rangos hormonales, pruebas de imagen, el test de TRH y ensayos de bioactividad de la TSH sérica de los pacientes. Ello nos llevará a identificar defectos genéticos en genes conocidos (actualmente muy escurridizos) y en otros por conocer, al investigar con las modernas técnicas genómicas pedigríes familiares con esta patología. *(El trabajo completo de esta revisión de los recientes hallazgos clínicos y genéticos conocidos hasta la fecha en HCC se refleja en el Anexo I de esta Tesis).*

# OBJETIVOS

*Marta García González. 2017.*

# *Hipotiroidismo congénito central: correlaciones clínico-genéticas e investigación de sus mecanismos moleculares*

# OBJETIVOS

1. Caracterización clínica y genética de una cohorte de 50 pacientes pediátricos y adultos con sospecha de hipotiroidismo congénito central (HCC).

    a. Discriminación etiológica del HCC en hipofisario o hipotalámico mediante perfil hormonal, test de TRH, estudio de imagen (Resonancia Magnética Nuclear) y de fenotipos asociados.
    b. Determinación *in vitro* de la bioactividad de la TSH en la cohorte de pacientes.
    c. Estudio de genes candidatos con el objetivo de identificar nuevas mutaciones en genes conocidos o genes que hasta ahora no se han descrito alterados en humanos, basándose en modelos animales de Hipotiroidismo Central, fundamentalmente murinos.
    d. Identificación de nuevos fenotipos asociados a estos defectos genéticos.

2. Análisis *in silico* de patogenicidad y verificación experimental de los defectos moleculares identificados y su relación causal con los casos clínicos estudiados (relación genotipo-fenotipo).

3. Secuenciación masiva por panel dirigido (Next Generation Sequencing, NGS) para identificar mutaciones en genes conocidos o variantes patogénicas en nuevos genes hasta ahora no implicados en HCC humano.

# RESULTADOS

*Marta García González. 2017.*

# *Hipotiroidismo congénito central: correlaciones clínico-genéticas e investigación de sus mecanismos moleculares*

# CAPÍTULO I

## Dinámica de secreción de la TSH y genética del Hipotiroidismo Central

El hipotiroidismo congénito central (HCC) es un trastorno infradiagnosticado causado por síntesis, secreción o bioactividad de la TSH, defectuosas por un fallo funcional de la célula tirotropa pituitaria o de su control hipotalámico. Las consecuencias clínicas del HCC y la genética subyacente son poco conocidas. Sólo se conocen cuatro genes cuyos defectos causan HCC en humanos: *TSHB*, *TRHR*, *IGSF1* y recientemente, *TBLX1*, todos expresados en células tirotropas de la hipófisis.

Los objetivos de este estudio se centraron en la investigación fenotípica y discriminación etiológica a través del test de TRH largo y de los antecedentes genéticos del HCC en una gran cohorte de pacientes pediátricos y adultos con hipotiroidismo central aislado o en el contexto de deficiencias combinadas de hormonas hipofisarias (DCHP).

La capacidad y la dinámica de la secreción de TSH se analizaron mediante el protocolo del test de TRH largo (3 h de duración) usando 3 parámetros para su evaluación: la capacidad de secreción total, la potencia de secreción y la dinámica de secreción de subida y bajada de la curva de TSH. La detección de mutaciones en genes conocidos implicados en HCC humano y otros candidatos en modelos animales y celulares se realizaron mediante métodos clásicos (PCR- secuenciación Sanger) o por secuenciación masiva (NGS) en un panel de 390 genes implicados en el eje hipotálamo-hipófisis-tiroides. Se investigaron las correlaciones fenotipo-genotipo.

El HCC se diagnostica tardíamente en niños (a los 7 años de edad), excepcionalmente en periodo neonata en países que utilizan programas de cribado neonatal basados en la TSH para el hipotiroidismo congénito. El análisis detallado de la secreción de TSH tras estímulo con TRH permitió la discriminación de 5 tipos de respuestas de secreción de TSH: 2 respuestas hipofisarias (P1 y P2), 2 respuestas hipotalámicas (H1 y H2) y una curva normal. La genética y los fenotipos clínico-radiológicos de los pacientes apoyan la existencia de estos grupos de respuesta a TRH. La respuesta P1 refleja el clásico fallo hipofisario, apoyado por la identificación de las mutaciones en *IGSF1*, *TRHR* y *POU1F1*. La respuesta P2 es una variante de P1 con dinámica de secreción retrasada, que no recupera los niveles basales de TSH al final de la prueba. La respuesta H1 representa el fallo hipotalámico clásico con secreción exagerada de TSH y dinámica de liberación lenta. Un correlato clínico para H1 es una niña con hipotiroidismo hipotalámico que asocia un fallo hipotalámico en el control de la actividad de la grasa parda, conduciendo a un hipermetabolismo energético. Las respuestas H2 son una variante de H1 y muestran un marcado retraso en el pico de secreción de la TSH. Curiosamente, todos los pacientes estudiados con DCHP y el "síndrome de tríada" por resonancia magnética (hipoplasia hipofisaria, tallo interrumpido y neurohipófisis ectópica) presentaron este tipo de curva de secreción. Se ha identificado por primera vez una hipoplasia hipofisaria e hipotalámica en una niña, lo que apoya nuestra sugerencia de que la respuesta H2 podría ser el reflejo de un defecto “mixto” por malformaciones hipotálamo-hipófisarias infradiagnosticadas. Las respuestas normales (N) no excluyen el hipotiroidismo central en un paciente, como revela la identificación de una mutación en TRHR (sutil pero) patogénica en una familia consanguínea de etnia gitana.

La NGS reveló nuevos genes candidatos interesantes en el HCC humano con funciones fisiológicas en la regulación transcripcional de los genes *TSHB* y/o *TRH* (*RXRG, NR4A1, ZFHX3*) y co-represores o co-activadores de la función del receptor de la hormona tiroidea (TR) dentro del complejo NCoR- SMRT (*NCOR1, NCOA1, NCOA3*) cuya participación definitiva en el HCC humano justifica amplios estudios de segregación familiar y demostraciones funcionales *in vitro* de su naturaleza patogénica.

En conclusión, el hipotiroidismo central puede ser diagnosticado clínicamente por una alta sospecha clínica por parte de los clínicos. Los pacientes pueden beneficiarse de la clasificación etiológica después de un análisis detallado del tipo de secreción de TSH tras estimulación con TRH, que se podría aplicar a las correlaciones fenotipo-genotipo cuando la identificación (inicial, pero todavía pobre) mediante NGS de genes candidatos para HCC se implemente en el diagnóstico clínico en un futuro próximo, especialmente cubriendo el hipotiroidismo central de origen hipotalámico, todavía huérfano desde la perspectiva molecular.

# TSH secretion dynamics and genetics of Central Hypothyroidism

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## Abstract

Central congenital hypothyroidism (CCH) is an underdiagnosed disorder caused by defective synthesis, secretion or bioactivity of TSH reflection the functional failure of the pituitary thyrotropic cell or its hypothalamic control. The clinical consequences of CCH and underlying genetics are poorly understood. Only four genes are known whose defects cause human CCH: *TSHB, TRHR, IGSF1* and recently, *TBLX1*, all expressed at the pituitary thyrotropes.

**Aim:** To investigate the clinical phenotype, etiological discrimination through the long TRH test and the genetic background of CCH in a large cohort of pediatric and adult patients with isolated central hypothyroidism or in the context of combined pituitary hormone deficiencies (CPHD).

**Methods:** TSH secretion capacity and dynamics were analyzed using the long (3 h lasting) protocol of the TRH test and 3 parameters: total secretion capacity, potency of secretion and timing of the rising and falling parts of the TSH curve. Mutation screening in known genes involved in human CCH and other candidates in animal and cell models were performed through classical (PCR-Sanger sequencing) methods or by targeted next generation sequencing (NGS) in a panel of 390 genes of the hypothalamic-pituitary-thyroidal hormone axis. Phenotype-genotype correlations were investigated.

**Results**: CCH is a late diagnosis in children (at mean 7 years of age), with some exceptional neonatal diagnoses in countries using TSH-based screening programs for congenital hypothyroidism. Detailed analysis of the TRH-stimulated TSH secretion allowed discrimination of 5 types of TSH secretion responses: 2 pituitary responses (P1 and P2), 2 hypothalamic responses (H1 and H2) and a normal curve. Genetics and clinical-radiological phenotypes of patients render support the existence of these groups at the TRH response. *P1 response* reflects the classical pituitary failure, supported by the identification of mutations on *IGSF1, TRHR* and *POU1F1*. *P2 response* is a P1 variant with delayed secretion dynamics, not recovering basal levels of TSH at the end of the test. *H1 response* is the classical hypothalamic failure with exaggerated TSH secretion and slow clearance dynamics. A clinical correlate for H1 is a girl with hypothalamic hypothyroidism and associated hypothalamic failure in the control of brown adipose tissue activity leading to emaciation through energy hypermetabolism. *H2 responses* are a variant of H1 showing a dramatic delay in the peak TSH secretion dynamics. Interestingly, all patients studied with CPH and the “triad syndrome” at MRI (pituitary hypoplasia, stalk and ectopic neurohypophysis) presented this type of secretion curve. Identification of hypoplasia of the pituitary and the hypothalamus for the first time in one girl

lead to our suggestion that H2 response could be considered a “mixed hypothalamic-pituitary” defect with (under-detected) hypothalamic malformations. Normal (N) responses do not exclude central hypothyroidism in a patient, as revealed by identification of a TRHR (subtle but) pathogenic mutation in a Roma consanguineous family.

NGS revealed interesting novel candidates for human CCH with either physiological roles in transcriptional regulation of *TSHB* and/or *TRH* genes (*RXRG, NR4A1, ZFHX3*) and co-repressors or co-activators of the TR (thyroid hormone receptor) function within the NCoR-SMRT nuclear protein complex (*NCOR1, NCOA1, NCOA3*) whose definitive involvement in human CCH warrants wide familiar segregation studies and *in vitro* functional demonstration of their pathogenic nature.

**Conclusions**: Central hypothyroidism can be clinically diagnosed through high clinical suspicion from clinicians. Patients can benefit from etiological classification following a detailed analysis of the TSH secretion type after TRH stimulation, which could be further applied to phenotype-genotype correlations when (initial, but still poor) identification of the genetic background of the disorder advances through (targeted-, exome wide) NGS implementation to clinical diagnosis in the near future, especially covering the hypothalamic central hypothyroidism, still orphan from the molecular perspective.

## Introduction

Central congenital hypothyroidism (CCH) is an underdiagnosed disorder due to low synthesis, secretion or biopotency of thyrotropin (TSH) which fails to properly stimulate an otherwise normal thyroid gland. (Persani, 2012). In most countries, neonatal screening programs for congenital hypothyroidism do not detect failures of the central regulation of thyroid hormone production since detection is based in TSH elevations over a given cut-off threshold (García M *et al.*, 2014, Annex I of this Thesis). Since CCH cannot be detected at birth in most countries, it is usually diagnosed in children through clinically mild hypothyroidism, short stature, poor school performance or lethargy, and sometimes through co-existence with other pituitary hormone deficiencies. Despite common beliefs, CCH at birth is not always mild. Actually, recent reports from countries screening for the disorder indicate that 55% of CCH cases present severe or moderate hypothyroidism at birth (Zwaveling-Soonawala N *et al.*, 2015). Therefore, severity of CCH should not be underestimated (Zwaveling-Soonawala N *et al.*, 2015). The important role of thyroid hormones during postnatal brain and general development and childhood applies to CCH cases. But additionally, serious acute complications that may derive from the coexistence of other pituitary deficiencies makes neonatal screening and diagnosis of CCH a cost-beneficial measure for outcome of the disorder and the general health outcomes of these patients (van Tijn DA *et al.*, 2005).

The use of the TRH test started from the 70's for diagnosis of central hypothyroidism, but also for confirmation of subclinical hypothyroidism, due to the low sensitivity of hormonal determinations then available (Ormston BJ *et al.,* 1971). From the 90's, sensitive methods (of $2^{nd}$-$3^{rd}$ generation) for TSH determination led to the abandonment of the test for the second indication, remaining however important in the etiological differentiation between secondary and tertiary hypothyroidism (Faglia G, 1998). Furthermore, the TRH test has been widely used in the diagnosis of a variety of pituitary disorders in adults and children such as pituitary tumors, Sheehan syndrome, empty sella, combined pituitary hormone deficiencies (CPHD) and pituitary resistance to thyroid hormones (Faglia G *et al.,* 1973; Sarne DH *et al.*, 1990).

The TRH test classically describes two main TSH responses associated with pituitary (blunted response) or hypothalamic failures (exaggerated and sustained response) (Costom BH *et al.*, 1971). In the past, interpretation of the outputs of the test raised controversy arguing the existence of “hypothalamic” TSH responses in patients with (allegedly isolated) pituitary defects (Mehta A *et al.*, 2003; Crofton PM *et al.*, 2008). In contrast, van Tijn et al. investigated in detailed the relative potency and dynamics of TSH secretion calculating TSH ratios at

different time points within the rising (peak/basal) and falling (final/basal) parts of the TSH curve after TRH stimulation, and found them discriminative (van Tijn DA *et al.*, 2008B). However, they designed a new protocol to maximize the TRH test performance in which TSH secretion was analyzed during 180 minutes (and 7 time points) after TRH administration, and applied it to the early etiological diagnosis of patients positive at the Dutch screening program for CH, which is based in a T4-TSH-TBG method in that country (Verkerk PH *et al.*, 2014). Most CH babies undergoing TRH test presented associated (combined) pituitary hormone deficiencies (CPHD) (van Tijn DA *et al.*, 2008A).

They could discriminate 3 well-characterized types of TSH response to TRH: type 0 (normal), type 2 (pituitary defects) and type3 (hypothalamic defects) in 25 patients, classified as false positives at screening (not confirmed CCH in serum) (n= 6), CPHD with pituitary responses to TRH (n= 4) and CPDH with hypothalamic responses at the test (n=9). Since other basal and hormone dynamic hormone tests were also performed (van Tijn DA *et al.*, 2007; van Tijn DA *et al.*, 2008A) most patients were shown to have CCH in the context of CPHD, and only 5 patients presented isolated TSH deficiency with pituitary response to TRH (van Tijn DA *et al.*, 2008B).

Aiming the most complete characterization of our patients we investigated their TSH responses to TRH in the 180 min-long test, their TSH/T4 ratios visualized using the Dietrich´s dynamic model (Dietrich JW *et al.*, 2012) and, in selected cases, the experimental bioactivity of their serum TSH through formal (non-routine) TSH bioactivity cell-based assay (García M *et al.*, 2017A, Chapter II of this Thesis). Since TRH stimulus at the test not only enhances secretion of TSH but also its biopotency through glycosylation, both pre- and post-TRH stimulation sera at the TRH test were used (García M *et al.*, 2017A, Chapter II of this Thesis).

Defects in only four genes are currently known to cause central hypothyroidism: *TSHB* (encoding the B-subunit of the TSH glycoprotein hormone), *TRHR* (encoding the specific 7-transmembrane domain pituitary receptor for hypothalamic TRH), *IGSF1* (encoding a protein regulating the expression of TRHR in pituitary thyrotropes), and the recently identified *TBL1X* (encoding a subunit of the nuclear NCoR-SMRT regulatory protein complex, active at the *TSHB* promoter) (García M *et al.*, 2014; Heinen CA *et al.*, 2016; García M *et al.*, 2017A; Chapter II and Annex I of this Thesis). However, central regulation of thyroid hormone synthesis involves complex processes in which many transcription factors influence the activity of the *TSHB* promoter (as POU1F1, GATA2, NR4A1), nuclear co-activators and co-repressors of the thyroid hormone and retinoid X receptor (TR, RXR) actions (as SRC1 or members of the NCoR-SMRT complex) and other proteins participating in pituitary organogenesis, including thyrotropes

(like PROP1, LHX4, LHX3, SOX2, SOX3, PITX1, PITX2, HESX1) act in a coordinated manner over time and space ensuring the correct development, maturation and function of the pituitary-thyroid axis and thyroid hormone synthesis (García M *et al.*, 2014, Annex I of this Thesis).
The detailed analysis of the TSH curve proposed by van Tijn for the TRH test inspired the present study aiming the best possible discrimination of types (and subtypes) of TSH response curves of -mainly *isolated*- central hypothyroidism through the 180 min-lasting TRH test, followed by the identification the underlying genetic causes of the disorder allowing investigation of phenotype-genotype correlations.

## Materials and Methods

### *Patient's cohort*

This is a retrospective study of a cohort of 50 pediatric and adult patients investigated for suspicion of Central Hypothyroidism. Patients were collected from 2011 to 2017, from a net of 16 hospitals in Spain. Two patients were referred for genetic study from Cyprus and one from Mexico. The majority of patients were from Caucasian origin. Informed consent for genetic studies was obtained from all index patients and their families, according to protocols followed the referral hospital where the patients were most clinically followed.

Patients were clinically (hormonally, morphologically) phenotyped. Hormone profiles (thyroid and other pituitary axes) were studied in all patients. The TRH test was performed in 29 patients (58%) and brain magnetic resonance imagen (MRI) was available in 30 patients (60%). Thyroid volume was determined by ultrasounds in most of patients. Patients were recruited following one or more of these inclusion criteria: [1]low or borderline-low FT4 (defined as >20% decrease over the lower limit of normality (Alexopoulou O *et al.*, 2004)) along with low, normal or slightly elevated TSH (< 10 mIU/L (Turan S *et al.*, 2015)), [2]low TSH, [3]low FT4 in the context of CPHD, and [4]syndromes known to associate CCH. Patients presenting any of these exclusion criteria were rejected from the study: [1]clinical symptoms of hyperthyroidism, [2]familiar history of toxic multinodular goiter or solitary autonomous nodule, [3]Graves' disease or autoimmune thyroiditis, [4]offspring of women with Graves' disease, and [5]central hypothyroidism secondary to tumor-related surgery.

### ***Diagnosis of CH***

All patients (except 1 patient from Cyprus) were screened for congenital hypothyroidism by TSH-based program, implemented in Spain. TSH was determined for blood spot in a filter

paper (from heel puncture) of neonates between 2nd and 7th days of life by an immunofluorescence assay (DELFIA ®) and using TSH threshold ≥ 10 mIU/L. One patient was screened for hypothyroidism in Mexico by TSH-based program in which TSH is determined for blood spot from heel puncture in neonates between 2nd and 15th days of life, using TSH threshold ≥ 25 mIU/L for positivity.

One patient was diagnosed in Cyprus as neonate by a T4-based screening. Total thyroixine (TT4) and TSH were determined from 1990 to 1992, thence, only the TSH is measured by a solid fluoroimmumometric assay (DELFIA® Neonatal hTSH kit (Wallac Oy, Turku, Finland), using a TSH threshold >12 mIU/L.

***Hormone determinations***

TSH, total triiodothyronine ($TT_3$), free thyroxine ($FT_4$), anti-thyroid antibodies, cortisol, prolactin (PRL), follicle-stimulating hormone (FSH), luteinizing hormone (LH), testosterone and estradiol were determined with Quimioluminiscent immunoanalysis in Centaur XP (Siemens, Healthineers). ACTH, IGF-1 and IGFBP-3 were determined with Quimioluminiscent immunoanalysis in Immulite 2000 (Siemens, Healthineers). In Cyprus DPC's Coat-a-Count TT4 (a radioimmunoassay) and Coat-a-Count TSH IRMA (Immunoradiometricassay) kits were used according to manufacturers' instructions.

***TRH test***

TRH stimulation test was performed after four weeks L-T4 treatment withdrawal. Serum TSH and prolactin were measured at -15, 0, 15, 30, 60, 90, 120 and 180 min after intravenous administration of TRH (Protirelin, 7μg/kg body weight; max 200 μg).

TSH response to TRH administration was analyzed with five criteria: [3] Total Capacity defined by area under the curve (AUC), [1] Absolute Maximum Capacity (TSH peak) defined by TSH peak value, [2] Relative Maximum Capacity (TSH potency) defined by TSH peak/basal ratio, [4] dynamics of the TSH rising curve defined by TSH potency in time and [5] dynamics of the falling curve defined mainly by the TSH 180'/0' ratio.

An adequate TSH total capacity is defined by AUC of 1385 (± 452) taken of 95 controls (data not published) and an adequate TSH peak was defined as a peak concentration value greater than 15 mIU/l (Okuno A *et al.*, 1979; Rapaport R *et al.*, 1993; van Tijn DA *et al.*, 2008B). TSH potency was evaluated by TSH 15'/0' and 30'/0' ratios, according to van Tijn (van Tijn DA *et al.*, 2008B). The TSH rise dynamics was evaluated in the ascending part of the curve and normal

dynamic is defined as a TSH peak at 30 minutes or below. TSH fall dynamics was evaluated in the descending part of the curve by 30'/60', 30'/180' and 180'/0' TSH ratios, following van Tijn (van Tijn DA *et al.*, 2008B).

TSH bioactivity was measured in a cell based assay using dilutions of the patient´s serum collected at the beginning and end of the TRH test, as reported (García *et al.*, 2017A, Chapter II of this Thesis). Indirect measure of TSH bioactivity was calculated through the % increase of serum FT4 180 minutes after TRH administration, as reported (Yamada M & Mori M, 2008).

The model of Dietrich *et al* was used to evaluate dynamics of thyroid hormones in patients with CCH. The model is based on the comparison of conventional univariate reference ranges for TSH and FT4 (red square) and a bihormonal reference region (green area) from nonlinear modelling of thyroid homeostasis (Dietrich JW *et al.*, 2012).

***Genetic studies***

Patients referred to genetics laboratory between 2011 and 2015 were studied by polymerase chain reaction (PCR) and Sanger sequencing. Complete coding regions of different candidate genes for CCH were amplified by PCR using appropriate primers flanking each exon: three candidate genes for isolated CCH (*TSHB*, *TRHR* and *IGSF1*), five candidate genes for combined pituitary hormone deficiencies (*LHX3, LHX4, SOX3, POU1F1*) and other four genes related to regulation of hypothalamus-pituitary axis (*TRH, PITX1, PITX2* and *NR4A1*) but with no mutations reported so far in human CCH. PCR products were purified and directly sequenced on an automated DNA sequencer (3100 Genetic Analyzer, Applied Biosystems). After implementation of next generation sequencing (NGS) in 2016, using NextSeq 500 (Illumina) platform, patients were studied through a panel of own design including 390 genes involved in the hypothalamus-pituitary thyroid axis physiology in humans, animal models and cell models (*TiroSeq V.1*). The panel captures the coding sequence and flanking intronic regions of a list of genes which is available upon request (including the recently identified *TBL1X* gene associated with CCH). Currently, we are retrospectively including patients referred to laboratory before 2016, in the NGS panel. To date, a total of 19 patients (38%) of our cohort were studied. Variants identified were filtered through criteria for prevalence in general population (MAF < 1%) in different databases for genomic variants (dbSNP, 1000 Genomes Project, The Exome Aggregation Consortium, Human Gene Mutation Database, ClinVar) and different *in silico* predictors (CADD, Sift, Polyphen2, MutAssesor, Fathmm, VEST).

Comparative Genomic Hybridization (CGH)-Array was performed in patients with suspicion of deletions or duplications, using two different platforms according to clinical suspicion:

[1]CytoSNP-850K BeadChip (Illumina), containing 850,000 selected single nucleotide polymorphisms spanning across the genome. This high density of SNPs enables high-resolution analysis for discovery of significant chromosomal aberrations. [2]Customized whole genome microarray, KaryoArray®v3.0, covering more than 380 clinically relevant regions of genomic imbalance was used in an Agilent-based high resolution 8x60K format, containing 62,976 distinct probes. Array experiments were performed as recommended by the manufacturer (Illumina, San Diego, California, USA or Agilent, Technologies, Santa Clara, California, USA, respectively).

## Results

### *Phenotypic characterization*

*Clinical diagnosis*

In this study we clinically characterized a cohort of 50 patients with suspicion (and later confirmation) of central hypothyroidism. Fifty percent of patients were female. Thirty-four patients were in the pediatric age (68%) with an average age at diagnosis of 7 years (7 days-16 years of age) while 14 were adults (28%) of 35 years mean age (18-60 years). We identified two consanguineous families (4%): one from Moroquian descent (Patient #3) and other of Roma origin (# 25).

Patients were not detected by TSH-based neonatal screening program implemented in Spain or Mexico. One patient (Patient 30) was detected by T4-based screening trial used in Cyprus during 1990 to 1992.

Six patients (12%) were detected as neonates (<1 month): a male (# 1) at 15 days of life with myxedematous face, protruding tongue and lethargy, a female (# 3) at 7 days of life presenting hypoglycemia, coarse face, mild hypertelorism and femur shortening, a male (# 31) at 15 days of life with severe jaundice and family history of CCH (his brother was detected by T4-screening in Cyprus), a male (# 32) at 4 days of life with jaundice, hypoactivity and poor breast feeding, a female (# 41) of 11 days of life with respiratory distress, and a male (# 45) in his first days of life with unavailable clinical information. Three patients (6%) were diagnosed in the first months of life: two females (# 5 and # 28) at 3 and 5 months of age, respectively, both with congenital cardiopathy and another female (# 50) at 10 months of age because studied febrile episodes and nervousness.

Seventeen patients (34%) were diagnosed during infancy (1-10 years of age) and fifteen patients (30%) in adolescence (11-16 years of age), for different reasons including CPHD, short stature, overweight, poor school performance.

*Associated features*

Central hypothyroidism has been associated with a variety of features and syndromes most of them of unknown genetic base. Five patients associated macroorchidism, two developmental disorders, psychomotor retardation and/or language delay, two patients with epilepsy and two patients with cardiopathy. Dysmorphic features, attentional-deficit and hyperactivity disorder, autism, subfertility and mental retardation are other features associated in our patients with CCH. Moreover, two patients presented Shapiro syndrome characterized by recurrent episodes of hypothermia, sweating and chill associated with central hypothyroidism of hypothalamic origin, as described (Arkader R & Takeuchi CA, 2008). One patient presented Alstrom syndrome with retinopathy, hyperinsulinism, deafness, hypertriglyceridemia, juvenile nephronophthisis and obesity associated with central hypothyroidism, as described (Charles SJ *et al.*, 1990). Finally, one patient presented Mayer-Rokitansky-Küster-Hauser (MRKH) syndrome, which was previously associated in the literature in one patient with subclinical hypothyroidism (Gorgojo JJ *et al.*, 2002).

*Etiological classification of CCH by the TRH test*

At clinical diagnosis, 90% of patients presented hypothyroidism (mean FT4 of 9.05 pmol/L, Normal: 11.6-21.9 pmol/L) with low, normal or slightly elevated TSH (mean TSH of 3.4 mIU/L, Normal: 0.5-4.7 mIU/L). The other 10% of patients presented euthyroid hypothyrotropinemia (mean TSH 0.47 mIU/L , mean FT4 15.3 pmol/L).

The long (3 hours) TRH test was used as a tool to identify the origin of central hypothyroidism (hypothalamic and pituitary). The TRH test was performed in 29 patients (58%) of which 22 presented isolated TSH deficiency (76%) and 7 with CPHD (24%). Twelve patients (41%) showed a reduced TSH secretion under TRH stimulation, with reduced total capacity (AUC) and TSH peak below 15 mIU/L, but normal TSH rise dynamics (TSH peak ≤30 minutes) consistent with pituitary type response (van Tijn DA *et al.*, 2008B) (Figure 1A). However, two different groups in this category can be clearly distinguished: the type P1 response with low total capacity (mean AUC = 422), low TSH peak (mean TSH peak value = 3.35 mIU/L) and low TSH potency (mean TSH peak/basal = 3.65), but normal dynamics of TSH rise and fall with a total recuperation of basal TSH levels at 180 minutes (mean TSH 180'/0' = 1); and the type P2

response with low total capacity (mean AUC = 1097), low TSH peak (mean TSH peak value = 8.40 mIU/L) but high TSH potency (mean TSH peak/basal = 12.11), and altered fall dynamics with a sustained TSH response that does not recover basal levels even 3 hours after stimulation with TRH (mean TSH 180'/0' = 2.38) (Figure 1A).

Ten patients (34%) showed an elevated TSH secretion, with a remarkably elevated total capacity and TSH peak above 25 mIU/L and which is incapable to recover basal levels 3 hours after TRH stimulus, described as a hypothalamic response by van Tijn (van Tijn DA *et al.*, 2008B) (Figure 1B). Within this two different types of responses can be distinguish: type H1 with an exaggerated total capacity (mean AUC = 4677), TSH peak (mean TSH peak = 40.93 mIU/L) and potency (mean TSH peak/basal = 10.92) and with normal TSH rise dynamics but with slow recovery of TSH, which cannot reach basal levels 3 hours after TRH stimulation (mean TSH 180'/0' = 1.97); and type H2 with high total capacity (mean AUC = 3643) and TSH peak (mean TSH peak = 25.47 mIU/L) but normal TSH potency (mean TSH peak/basal = 5.10), delayed TSH peak time (at 60 minutes of median) and sustained TSH response 3 hours after TRH stimulus with TSH above the basal levels (mean TSH 180'/0' = 2.63) (Figure 1B).

Five patients presented normal TSH response to TRH in the test. Normal total capacity (mean AUC = 1781), TSH peak (mean TSH peak = 21.21) and TSH potency (mean TSH peak/basal = 6.41) are represented in the ascending part of the curve, with a normal dynamics of TSH rise. Dynamic of TSH fall in the descending part of the curve is also normal with completely recovery of TSH to basal levels (mean TSH 180'/0' = 1.10) (Figure 1C).

In twenty-two patients (76%) prolactin response was studied at the TRH test (data not shown). Only three patients presented null prolactin response to TRH stimulus, all with CPHD and two of them with hypothalamic type H2, presenting the triad of pituitary malformations on MRI.

FT4 before and after 180 minutes of TRH administration were available in 18 patients. The percentage increase of serum FT4 at the end of the test was used as an indirect measure of TSH bioactivity and there were not significant differences between pituitary, hypothalamic and normal responders ($p>0.2$). Despite of the variety of FT4 increases observed within each group, there is a trend toward lower bioactivity in patients with hypothalamic responses compared with those of pituitary response (Supplemental Figure 1).

*Hormone profiles in patients etiologically classified by the TRH test*

TSH, FT4 and TT3 levels in all patients were grouped upon their individual response classified at the TRH test (P response = pituitary, H response = hypothalamic and N response = normal) and compared that of those not submitted to the test (No TRH test) (Figures 2A, B and C).

TSH levels for pituitary responders (median = 0.51 mIU/L) were significantly lower from those of patients with hypothalamic (median = 4.48 mIU/L) and normal responses (median = 2.06) in the test, with a p value of $2.4 \times 10^{-5}$ and $4.2 \times 10^{-4}$, respectively. TSH ranges of CCH patients not subject to the test comprise all ranges of all groups, from pituitary to hypothalamic responses, probably due to heterogeneous origin of the disorder (Figure 2A).

Median of FT4 levels in all groups was under lower limit of the reference range, compatible with hypothyroid state. There were no significant differences in FT4 levels between pituitary (median = 11.1 pmol/L), hypothalamic (median = 10.1 pmol/L) or normal (median = 9.5 pmol/L) responses to the test, with a p value above 0.2. Patients no submitted to the TRH test were all hypothyroid and some of them with a severe decreased of FT4 below 4 pmol/L (normal range = 11.6-21.9 pmol/L) (Figure 2B).

TT3 levels available were in the normal range in all patients, excepted two with CPHD and without TRH test. No significant differences were observed between pituitary (median = 1.06 ng/ml), hypothalamic responses (median = 1.32 ng/ml) or patients without TRH test (median = 0.78), with a p value above 0.1 (Figure 2C).

The comparison of TSH/FT4 medians of each group of TSH response showed significant differences between pituitary and hypothalamic or normal responses with a p value of $5.2 \times 10^{-4}$ and $1 \times 10^{-4}$, respectively (Figure 2D). However, a more dynamic way of studying the behavior of the pituitary-thyroid axis is through the *Dietrich et al* model.

*Mapping of TRH-test classification of CCH patients at the Dietrich's model.*

Hormone values of each patient were individually plotted in the graphical model proposed by *Dietrich et al.* representing the dynamic relation between TSH and FT4 in order to confirm or rule out failures of the thyrotropic function (Dietrich JW *et al.*, 2012). And it is generated to discriminate pituitary defects from non-pituitary defects of the thyroid hormone axis.

All dots representing each patient fall outside red square (defined by normal range values for TSH and FT4) and green area (defining dynamic relation of both parameters in the model), indicating altered thyroid homeostasis.

Patients with pituitary P1 and P2 responses fell below 2% of normal pituitary response of the Dietrich *et al.* model, even those euthyroid but hypothyrotropinemic patients also have an altered hormonal relation, probably as a consequence of a deregulation of thyroid hormone axis (Figure 3A). Patients with hypothalamic H1 response fell above 2% of pituitary response but below 2% of thyroid response of the Dietrich *et al.* model, into areas of hypothyroidism, with the exception of two patients who overlaps with those with pituitary defects. On the other hand, 2/4 with type H2 response fell below 2% of both pituitary and thyroid normal responses of the Dietrich *et al.* model, suggesting a severe defect (Figure 3B). Most of patients with normal TSH response to TRH stimulus are in the intersection of 2% of pituitary and thyroid normal responses of the Dietrich *et al.* model, located in zone of overlap between pituitary and hypothalamic responses (Figure 3C). The remaining patients without TRH test fell in all areas where patients with pituitary, hypothalamic and normal responses are located (Figure 3D). The model clearly differentiate patients with CCH in three groups, classifying them in the same sense as the TRH test, supporting the use of both tools in the diagnosis of hypothyroidism of central origin.

*Isolated TSH vs combined pituitary hormone deficiency (CPHD)*

Thirty-four of fifty patients (68%) presented isolated TSH deficiency, whereas sixteen patients (32%) presented other pituitary axes disturbed leading to CPHD. The majority of these patients presented TSH combined with GH deficiencies (87%) followed by gonadotropins (FSH and LH) (62%), ACTH (37%) and PRL (19%) deficiencies. All patients were hypothyroid (mean FT4 = 8 pmol/L) and with TSH levels between severe hypothyrotropinemia (<0.005 mIU/L) and mild hyperthyrotropinemia (10.48 mIU/L), with the exception of one patient who was euthyroid but with low levels of TSH. Two patients presented TT3 levels below the normal ranges, being the only patients of our cohort with this hormonal feature.

*Brain MRI*

Study of pituitary and other brain structures was performed by magnetic resonance imaging (MRI) in twenty-nine patients (58%).

Sixteen patients presented isolated TSH deficiency (55%), fourteen with normal hypophysis (Figure 4A) and two with pituitary hypoplasia (Figure 4B). These patients presented all types of responses at the TRH test, excepted for H2 response, which did not observed in these patients (Supplemental Table 1).

Thirteen patients presented CPHD (45%) with all combinations of pituitary malformations. Three of them presented the classic triad malformation of hypoplastic adenohypophysis, ectopic neurohypophysis and absence of pituitary stalk, presenting hypothalamic H2 response at the TRH test (Figure 4C). One patient presented hypoplastic adenohypophysis and ectopic neurohypophysis, with also hypothalamic H2 response. One patient presented hypoplastic adenohypophysis and absence of stalk and other one with only neurohypophysis ectopia. Three patients with pituitary hypoplasia and other three with normal pituitary location and size, all of them with pituitary responses at the TRH test. Interestingly, one patient presented both adenohypophysis and hypothalamus hypoplasia associated with TSH and GH deficiency and presenting hypothalamic H2 response at the TRH test. Giving anatomic background, this patient presented mixed hypothalamic and pituitary defect (Figure 4D).

Three patients presented Arnold-Chiari type I malformation associated with the triad or only anterior pituitary hypoplasia in two patients with CPHD and with normal pituitary in one patient with isolated TSH deficiency, respectively.

***Genetics***

*1. Genetic studies*

In our cohort of 50 CCH patients, we identified 16% of patients with variants in the four genes previously associated with central hypothyroidism (*TSHB, TRHR, IGSF1* and *TBL1X*), and one patient with a defect in a gene associated with CPHD (POU1F1). Moreover, 20% of patients presented variants in genes previously not associated with CCH in humans, but involved in pituitary or hypothalamic regulation in animal or cell models. Three genes are involved in the positive regulation of *TSHB* expression: *NR4A1* and *ZFHX3* transcription factors and *NCOR1*, the nuclear corepressor of T3 action (Nakajima Y *et al.*, 2012; Qi Y *et al.*, 2008; Costa-e-Sousa RH *et al.,* 2012), and two further genes are related to negative control exerted by T3 over *TSHB* and *TRH* genes (*NCOA1* and *RXRG*) (Takeuchi Y *et al.*, 2002; Brown NS *et al.*, 2000).

Finally, we identified defects in genes expressed in pituitary and/or hypothalamus but poorly studied in the context of TSH deficiency: a subunit of the transcriptional repression NCOR1/SMRT complex (*GPS2*), and nuclear receptor co-activator (*NCOA3*).

<u>Gene defects yet associated with CCH</u>

We identified 6 pathogenic variants (in 7 patients) in three genes whose defects are previously described to cause CCH: three in *IGSF1,* two in *TBL1X* and one in *TRHR*.

We identified defects in ***IGSF1***, a gene expressed in pituitary thyrotropes and gonadotropes, in 4 patients. Two males (patients #1 and #32) have complete hemizygous deletions of *IGSF1* (Supplemental Figure 3A). Both cases were clinically follow-up from newborn to date (adult and child, respectively) with severe congenital hypothyroidism detected in early postnatal period in Spain and Mexico, respectively, and development of macroorchidism from 3 years of age (García M *et al.*, 2017A, Chapter II of this Thesis). One of them presented over-secretion of FSH at neonatal "mini-puberty" and both showed a premature stimulation of FSH by gonadotropin releasing hormone (GnRH) in childhood, but without increase in testosterone, ruling out precocious puberty. The detailed study of IGSF1 expression in the pituitary (thyrotropes and gonadotropes), the oversecretion of FSH at mini-puberty and the increase in Inhibin B with no effect on reducing FSH levels lead to a pathophysiological model of the disorder (Chapter II of this Thesis), including the proposal of IGSF1 as a pseudoreceptor for Inhibin-B in gonadotropes.

Two brothers (#30 and #31) were identified with a complex hemizygous INDEL mutation consisting in a duplication of seven base pairs (CAATAAG) in intron 17 of *IGSF1* followed by a deletion of 739 bp, including the complete sequence of exon 18 and 9 bp of exon 19 of the gene (c.3488-59_3775delinsCAATAAG) (Chapter III of this Thesis). Both siblings were diagnosed of CCH in early postnatal age (one of them through T4-based screening) with low T4 (4 and 0.44 pmol/L, Normal: 11-25 pmol/L), normal TSH (both with 2.7 mU/L, Normal: 0.5-4.7 mIU/L) and with TSH-FT4 relation outside green normal area in the Dietrich *et al.* model (Figure 5A), and L-T4 treatment was implemented. Interestingly, at reevaluation (withdrawal of levo-thyroxine for 1 month) both siblings showed a remarkable hyperthyrotropinemia (>150 mIU/L) and severe thyroid hypoplasia was confirmed, typical of a primary (thyroid gland) defects. This prompted us to investigate whether IGSF1 could be possibly involved in thyroidal growth. Even when this theory should be tested in embryological experiments, we here show for the first time that IGSF1 is expressed in follicular cells of the thyroid, offering good grounds for the hypothesis by which IGSF1 defects would lead to mixed pituitary and primary hypothyroidism of variable degrees (Chapter III of this Thesis).

We also identified one male (#25), born from consanguineous parents, with a homozygous pathogenic mutation in ***TRHR*** (p.Ile131Thr). This mutation was not previously described in variant databases. Patient presented with moderate central hypothyroidism (TSH: 2.61 mIU/L, Normal: 0.27-4.2; FT4: 9.52 pmol/L, Normal: 10.9-25.7) with TSH-FT4 relation in the borderline of normal green area in the Dietrich *et al.* model at 8 years of age (Figure 5A). He was overweight but with normal stature. Interestingly, his mother, two brothers and grandmother

were heterozygous for the mutation and showed isolated hyperthyrotropinemia of central origin (TSH: 4.3-8 mIU/L). The pathogenicity of the mutation was demonstrated by functional studies showing it decreases TRH binding and TRHR-Gq coupling and signalling (García M *et al.*, 2017B, Chapter IV of this Thesis).

In ***TBL1X*** (Transducin Beta Like 1X-Linked) we identified defects in two males (# 2 and #33). Patient 2 carried a *de novo* hemizygous nonsense mutation, p.R339X, the most N-terminal mutation described so far in this gene. The boy presented mild central hypothyroidism (T4: 10.42 pmol/L with inappropriately normal TSH of 1.57 mIU/L) with TSH-FT4 relation outside normal green area of the Dietrich *et al.* model (Figure 5A). TRH test showed reduced TSH ("pituitary") and normal prolactin responses. Interestingly, associated features included hearing loss, attention-deficit and hyperactivity disorder, encopresis, relative macrocephaly and Arnold-Chiari type I malformation, conforming what we propose is the fully-blown "TBLX1 syndrome" probably caused by the truly functionally deleterious mutation known in the gene (Chapter V of this thesis).

Patient #33 with *TBL1X* defect had a complete hemizygous deletion in the context of a large (9.6 Mb) deletion in chromosome X (Xp22.33-p22.2) (Supplemental Figure 3B) with a poly-malformative syndrome affecting midline (central incisor in the palate), deficiency of other pituitary axes (TSH, GH, FSH and LH), bilateral cryptorchidism, nystagmus, mental retardation, autistic and attention-deficit behaviour and, interestingly, also macrocephaly. His TSH was inappropriately normal (2.42 mIU/L) for a low FT4 (4.76 pmol/L), suggesting TSH deficiency. Pituitary morphology and size were normal.

Defects in ***TSHB*** result in central hypothyroidism with typical recessive inheritance. In Patient 21 we identified the heterozygous variant p.Ser20Cys in the context of CPHD (all pituitary axes affected including triad pituitary malformation in MRI and hypothalamic type H2 response in the TRH test). This variant has not been described in databases (only EXAC showed a population frequency near to 0%) and *in silico* pathogenicity studies predicts it as a pathogenic mutation. Therefore we believe that is not involved on the phenotype and central hypothyroidism of the patient (classically, monoallelic *TSHB* mutations in the gene do not lead to disease), but it is worth mention given its pathogenic profile of the change (Table 1).

<u>Genes associated with CPHD</u>

We also identified defects in genes related to CPHD like ***POU1F1***. Patient #3 is a female, born to consanguineous parents, diagnosed at 7 days of life by a neonatal hypoglycaemia, coarse

face, mild hypertelorism and femur shortening, with remarkably low FT4 levels (4.38 pmol/L) and undetectable TSH (< 0.005 mIU/L). Moreover, the patient presented severe prolactin and GH deficiencies. MRI was normal and the TRH test showed null TSH (P1 type) and prolactin responses, typical of pituitary hypothyroidism (Figure 1A). Her TSH-FT4 relation falls in areas of severe hypothyroidism and hypothyrotropinemia at the Dietrich model, consistent with hypophyseal defect (Figure 5A). This patient, Moroquian consanguineous descent, carried the homozygous mutation p.R265W in *POU1F1* which has been previously associated with TSH, PRL and GH deficiencies and shown pathogenic in cellular *in vitro* assays (Supplemental Figure 2) (Bircan I *et al.*, 2001).

Patient #44 is a male diagnosed of hypopituitarism with TSH and GH deficiencies with short stature growth, truncal obesity and relative macrocephaly. The boy also presented scrotal and penis hypoplasia but normal descended testes. TRH test and pituitary MRI were not available. Using NGS, a nonsense heterozygous mutation in the *BMP4* gene has been identified (Personal communication from Dr. Campos-Barros *et al.*, 2017), associated with a *TSHR* defect (Supplemental Table 2).

Genes involve in positive regulation of *TSHB* expression

Mutations in *NR4A1*, *ZFHX3 and NCOR1*, genes expressed in thyrotropes, are not yet described in humans. Patients with variants in genes involved in the positive (either directly or indirectly) regulation of *TSHB,* as *ZFHX3*, *NR4A1* and *NCOR1* (Nakajima Y *et al.*, 2012; Qi Y *et al.*, 2008; Costa-e-Sousa RH *et al.,* 2012; Astapova I *et al.*, 2011), present very similar TSH-FT4 relation located at the areas of defective pituitary response upon the Dietrich´s model (Figure 5B). Patient 26 harbors a heterozygous *de novo* ***NCOR1*** variant (c.2182+1G>T) in a consensus nucleotide of the splice-site, not previously described very likely affecting the structure of the mature mRNA (Supplemental Figure 2). The patient, without familiar history of hypothyroidism, presented a mild central hypothyroidism diagnosed at 3 years of age by isolated TSH deficiency, attention-deficit hyperactivity disorder, language and hearing difficulties and microcephaly accompanied by a normal TSH response at the TRH test.

It is common that patients with defects in pituitary transcription factors present various hormone deficiencies. This is the case of patient 49, carrying a heterozygous variant in ***NR4A1*** gene (p.Arg563Gln), who presented additional FSH and LH deficiencies leading partial hypogonadism and subfertility in combination with TSH deficiency, but normal pituitary MRI (Supplemental Figure 2). NR4A1 is known to participate in *FSHB* and *TSHB* promoters (Nakajima Y *et al.*, 2012).

Finally, we identified variants in a transcription factor that stimulates *POU1F1* expression: ***ZFHX3.*** Patient 34 carried the p.Arg1108Cys variant in the heterozygous state, not previously described and predicted pathogenic by *in silico* programs (Table 1). The patient showed mild central hypothyroidism with normal-borderline FT4 levels (11.71 pmol/L) and normal TSH (1.14 mIU/L) and Mayer-Roktansky-Küster-Hauser (MRKH) syndrome. The mutation was inherited from her father (Supplemental Figure 2).

Yet another patient (#11) presented a variant in ***ZFHX3*** gene (p.3519_3526del) in combination with the variant p.Val170Ala in ***CXCL12*** gene, both in heterozygous state and neither described (Table 1). This patient was diagnosed of mild isolated CCH. He presented slightly reduced FT4 levels (11.20 pmol/L) and normal TSH (1.91 mIU/L). Pituitary MRI was not performed but the TRH test showed a pituitary TSH response (P1) and normal prolactin. TSH-FT4 relation falls within the green area of normality but outside red square (representing the reference ranges of thyroid hormones), indicating the mildness of this phenotype (Figure 5B).

Genes involve in negative regulation of *TSHB* expression

On the other hand, we identified variants not previously described in two genes involved in the negative regulation of *TSHB* expression by thyroid hormone: *NCOA1* (*SCR1*) and *RXRG*, coactivator of TR and partner of TR in functional RXR-TR heterodimers, respectively (Takeuchi Y *et al.*, 2002; Brown NS *et al.*, 2000). Patients (# 9, #12) showed a pituitary (P2) response in the TRH test and their TSH-FT4 relation fall in the typical area for pituitary defects, close to green area (Figure 5B). Patient 9 carried the heterozygous p.Pro510Thr variant in ***NCOA1*** gene, predicted as a pathogenic variant. This patient was diagnosed at 9 year of age with hypothyrotropinemia (TSH: 0.46 mIU/L) but normal FT4 leves (13.38 pmol/L). In follow-up, however, she presented borderline-low FT4 (11.5 pmol/L, normal: 11.6-21.9) and mild symptoms of fatigue, poor school performance. This girl has also short stature and any additional pituitary axes disturbed.

Patients #12 and #36 (mother and daughter) carried the variant p.Leu277Val in ***RXRG*** gene in the heterozygous state, predicted as a pathogenic variant (Table 1) (Supplemental Figure 2). Both patients present normal TSH (1.25 and 1.73 mIU/L, respectively) and low FT4 (10.17 and 11.45 pmol/L, respectively). Patient 36 also associated hyperinsulinism and ovaric functional hyperandrogenism with a probably polycystic ovary causing amenorrhea. Beside its essential role in T3-dependent regulation of TSH secretion together with TR (Brown NS *et al.*, 2000), RXR negatively regulates glucose-stimulated insulin secretion of pancreatic B-cells (Miyazaki S *et al.*, 2010).

Defects in other genes poorly studied, but associated with thyroid hormone regulation, were identified. A patient (#18) carrying a heterozygous variant in ***GPS2*** gene (p.Ala203Thr) (a subunit of NCOR1-SMRT complex) presented normal-high TSH (4.27 mIU/L) with slightly low FT4 (10.17 pmol/L). He also presents a heterozygous variant in the thyroglobulin (***TG)*** gene (p.Gly2548Cys), which may be influencing the normal-high TSH levels in this patient. Pituitary MRI was normal but the TRH test showed a hypothalamic H1 type response of the TSH and his TSH-FT4 relation falls in hypothalamic areas (Figure 5B). He also manifested short stature, psychomotor retardation, language delay and, interestingly, macrocephaly (all patients detected with defects in genes encoding components of the NCOR1-SMRT complex, present alteration in the brain-cranial size).

Finally, Patient 28 carried a missense heterozygous variant (p.Glu372Lys) in ***NCOA3***. This variant was not previously described, but it is predicted as a likely pathogenic mutation. The patient was diagnosed at 3 months of age with CCH presenting low FT4 (7.72 pmol/L) and normal TSH (1.84 mIU/L) and no other pituitary axes disturbed. Patient did not manifest symptoms of hypothyroidism and presents a congenital cardiopathy (atrial septal defect, ASD), spontaneously resolved. Pituitary MRI and the TRH test were normal. TSH-FT4 relation was in pituitary response areas, suggesting a pituitary defect (Figure 5B).

*2. Types of TSH response in different genetic pituitary defects*

Based on genetic results and comparing the different types of TSH response to TRH in the different patients. We observed that patients with defects in the classical genes associated with CCH showed the classical pituitary response P1 type. Thus, *POU1F1* is the most severe defect which a TSH response completely absent, followed by *IGSF1* complete deletion and *TBL1X* non-sense mutation which showed a reduced but present TSH response (Figure 6A). The missense mutation in *TRHR* caused a mild phenotype of CCH compatible with a normal but borderline TSH response observed (Figure 6B).

The only patient with a hypothalamic defect clinically confirmed is a girl with hypothyroidism associated with hypermetabolism. Basal energy expenditure was reduced at propranolol test by inhibition of the sympathetic nervous system (which is regulated by hypothalamic T3), and the TRH test showed hypothalamic H1 response, supporting the hypothalamic origin of the disease (Figure 6C) (Chapter VI of this Thesis).

On the other hand, we described a patient with hypothalamic and pituitary hypoplasia by MRI, associated with H2 TSH response at the TRH test, supporting a mixed hypothalamic and pituitary origin of this disease (Figure 6D).

Interestingly, in two patients with *IGSF1* and *TRHR* defects the TRH test was performed in more than one occasion, showing an improvement in the TSH peak in response to TRH in each stimulus through age (Supplemental Figure 4). This feature is more pronounced in the patient with *TRHR* defect, who increased the TSH peak with more potent stimulation (Supplemental Figure 4B).

## Discussion

The present study investigated the dynamics of TSH secretion and the genetic background of the largest cohort of central hypothyroidism reported so far. The cohort is unique for its large proportion of cases with *isolated* central hypothyroidism (68%), in which the systematic analysis of the TRH test is unprecedented. Indeed, TSH response to TRH has been basically investigated in central hypothyroidism in the context of CPHD (Mehta A *et al.*, 2003, van Tijn DA *et al.*, 2008B). In our cohort, patients were studied as children or adults, not as newborns (van Tijn DA *et al.*, 2008B), since most patients were born in countries using TSH-based screening methodologies for CH detection. Given that hormone failures in CPHD are normally asynchronous, a non-neonatal diagnosis of CCH in our cohort represents certainty for the diagnosis of true *isolated* hypothyroidism (van Tijn DA *et al.*, 2008B).

*Diagnosis and clinical presentation*

Suspicion of central hypothyroidism from clinical grounds may not be straightforward, and relies on subtle signs and symptoms of hypothyroidism. Therefore, pediatric CCH is frequently unnoticed or remains undiagnosed through adulthood. Short stature, poor school performance or lethargy are frequent circumstance leading to diagnosis in children (Persani, 2012 ; García M *et al.*, 2014), at first suggesting central neonatal hypothyroidism should be mild. However, a recent survey in The Netherlands (using a T4-TSH-TBG strategy for CCH detection at birth) showed that severe and moderate hypothyroidism represent 55% of babies, and their consequences should not be underestimated (Zwaveling-Soonawala N *et al.*, 2015).

In our cohort, mean age at diagnosis of pediatric patients was 7 years. Only 6 children were diagnosed in their 1$^{st}$ month of life: three babies presented hypoglycemia and respiratory distress, respectively, within CPHD (Patients 3, 41 and 45), but the other three had isolated CH (Patients 1, 31 and 32) (García M *et al.*, 2017A, Chapter II of this Thesis). Exceptionally, one patient from Cyprus was detected within a 2-year trial CH screening program using T4-based strategy (Patient 30). This represents 14% neonatal diagnosis in our cohort. In other CCH series, this percentage reached 28% (Mehta A *et al.*, 2003), however such cohorts included

basically children with CPHD, who presented neonatal morbidities derived from the other pituitary deficiencies (van Tijn DA *et al.*, 2008B). These data translate the difficulties to detect isolated CCH only from clinical skills in countries not benefitting from T4-based screening programs for the disorder. In adults, one woman was diagnosed of CCH in the context of CPHD for difficulties becoming pregnant. These patients could remain undiagnosed and suppose a risk for their future offspring. Pregnancy is a stage of life with hormonal requirements higher which can evolve in hypothyroxinemia. This gestation hypothyroxinemia can be missed by routine TSH evaluation of the thyroid axis during pregnancy aggravating the hypothyroid state of the fetus who may well have inherited the genetic defect from the mother (Pine-Twaddell E *et al.*, 2013).

Beside signs from other pituitary deficiencies in CPHD (hypoglycemia, respiratory distress episodes, micropenis) CCH may associate other clinical features helping diagnosis. Macroorchidism in a child (>P97 testicular size for age) (García M *et al.*, 2017A, Chapter II of this Thesis) should trigger suspicion *IGSF1* gene defects, including central neonatal hypothyroidism (Patient 1, 30, 31 and 32). Diagnosis of macroorchidism is difficult under 3 years of age, however, the gonadal axis may be overstimulated from birth in this disorder, which is detectable by increased FSH at neonatal minipuberty (García M *et al.*, 2017A, Chapter II of this Thesis). Alterations in cranial volume as macrocephaly (>3 SD head circumference for age and gender), relative macrocephaly (head size within normal range, but disproportionately larger head related to height/weight centiles of the child) or microcephaly (<3 SD head circumference for age and gender) was present in 4 children in the cohort, representing a prevalence of 8%. Interestingly, genetic investigation in those patients identified defects in components of the Nuclear Corepresor (NCoR)-Silencing Mediator of the Retinod and Thyroid receptors (SMRT) complexes modulating TSH-Beta expression at the pituitary like *TBL1X* (#2 and #33), GPS2 (#18) and NCOR1 (#26). Functional studies are warranted for the mutations identified in corepressors and coactivators, but cranial growth alterations in these patients points towards a common signaling pathway mediated by the NCoR-SMRT complex, active in brain-cranial development, and possibly involving Wnt-B-Catenin transcriptional signaling (Chapter V of this Thesis).

Finally, 12 children with isolated CCH (24%) and 2 children with CPHD (4%) associated neurological features like attention-deficit, hyperactivity, poor school performance, language or learning difficulties, epilepsy, autism or psychomotor retardation. No formal studies are available comparing psychomotor development of CCH infants with late diagnosis versus that of children early detected and treated by T4-based neonatal screening. However, CCH children

diagnosed after 3 months of age are described with psychomotor and intellectual delays when evaluated at 2–8 years suggesting significant benefits may derive from CCH detection at birth (Dacou-Voutetakis *et al.*, 1990; Bonomi M *et al.*, 2001; Baquedano MS *et al.*, 2010). Classically, neurological features of undiagnosed CH are all attributed to the T4 shortage affecting the correct maturation of the brain. While this assumption has strong scientific grounds from both clinical and experimental evidence, we propose that, at least for particular genetic defects associated neurodevelopmental disorders (e.g. *TBL1X* defects and autistic spectrum disorder), the derangement of specific neuronal-synaptic pathways in which the gene is also participating may contribute to this frequent association (Chapter V of this Thesis).

*Types of TSH response to TRH*

The TRH test consists in the i.v. administration of TRH, natural stimulus for TSH synthesis and secretion, and determination of the serum TSH concentration curve in a given lapse of time. Before availability of third generation TSH assays, it was used for confirmation of subclinical hypothyroidism and as nowadays, for discrimination between pituitary or hypothalamic (secondary or terciary) origins of central hypothyroidism (Faglia G *et al.*, 1973). The advent of sensitive methods for serum TSH determination made its first indication unnecessary and its role in the etiological investigation of CCH became controversial (Mehta A *et al.*, 2003), because pituitary and hypothalamic responses could be both present in patients with CPHD, a disorder allegedly pertaining the pituitary gland exclusively. However, in The Netherlands van Tijn *et al.* applied the TRH test in babies diagnosed with central hypothyroidism by the Dutch screening CH program, showing that it is useful in the etiological diagnosis of the disorder (van Tijn DA *et al.*, 2008B). Differences between the two studies included the use of two different forms of the TRH test.

Mehta and coworkers used the classical (short) form of TRH test and including 3 TSH determinations (basal, 15´, 30´ and 60') after TRH stimulus (Mehta A *et al.*, 2003). They essentially analyzed the (sufficient vs insufficient) capacity of the pituitary to secrete TSH, based on the TSH concentrations reached at the peak. In contrast, van Tijn *et al.* developed a (long) test lasting for 180 minutes and including 7 TSH determinations (basal, 15´, 30´, 45´, 60´, 120´ and 180´) (van Tijn DA *et al.*, 2008B). Therefore, they analyzed included not only the maximal capacity of TSH secretion (low, normal or exaggerated) but also the fine timing of the peak (including 45´) and, importantly, the dynamics of the rise and fall of TSH concentrations in serum, with the ratios between peak and basal TSH and between the final (180´) to basal TSH, respectively. In their opinion, not only the magnitude of the TSH peak, but also its timing and

dynamics of rise and fall of the TSH curve (peak/basal and 180´/basal TSH ratios) are essential to discriminate pituitary from hypothalamic responses, which they named "type 2 and type 3 responses", in contrast to the normal "type 0 response" (van Tijn DA *et al.*, 2008B).

Following van Tijn´s analysis we aimed to evaluate: the *total capacity* for TSH secretion (using area under de curve, AUC), the *absolute maximal capacity* (TSH peak value), the *relative maximal capacity or potency* (TSH peak/basal ratio) and the *dynamics* of TSH secretion throughout the test (Figure 7). Furthermore, free T4 were determined at 0´ and 180´, and % increased used as indirect measure of TSH bioactivity (Yamada M & Mori M, 2008). A *TSH bioactivity assay* was exceptionally performed using dilutions of patient´s sera if available (García M *et al.*, 2017A, Chapter II of this Thesis).

Following these 4 parameters, we were able to distinguish the 3 basic types of TSH responses to TRH: the *normal* (type 0), the *pituitary* (type2) and the *hypothalamic* (type3) responses of van Tijn, but additionally we could also discriminate 2 subtypes of pituitary (P1 and P2) and hypothalamic (H1 and H2) secretion failures (Figure 7).

**P1 response** is consistent with the classic **type 2** response described by van Tijn. These curves have low total capacity (AUC) and TSH potency but correct peak timing and dynamics of TSH fall with complete recovery of basal TSH levels at 180 minutes. In the model of Dietrich *et al*, TSH-FT4 relation of patients with this type of response is located below 2% of normal pituitary response and presenting significantly lower TSH levels than other groups, compatible with a defect of pituitary origin.

**P2 response** have decreased total capacity (AUC) and correct timing, overlapping with P1 responses, however showing elevated potency and disturbed (slow) TSH fall dynamics, with lack of recovery of basal TSH at 180 minutes. In the model of Dietrich *et al*, TSH-FT4 relation of patients with P2 response is located below 2% of normal pituitary response, but most of them above 97% of thyroid response. Some of these patients were euthyroid with low immunoreactive TSH of central origin, demonstrated in two patients with short stature and one of them in the context of GH deficiency and with pituitary hypoplasia. This hormone pattern was previously described in two children with short stature and the mechanism involve in maintaining euthyroidism despite of hypothyrotropinemia remains to be elucidated (Sato T *et al.*, 1989).

**H1 response** is characterized by an exaggerated total capacity for TSH secretion (AUC) and peak potency, reaching remarkably elevated serum TSH at normal timing (15 or 30 minutes)

but extremely slow dynamics of TSH fall leading to a “sustained” TSH secretion throughout the long (3-hour lasting) TRH test. In the model of Dietrich *et al*, TSH-FT4 relation of most patients is located above 2% of pituitary response but below 2% of thyroid response, into areas of hypothyroidism and with significantly elevated TSH levels respected pituitary patients.

**H2 response** is a variant of H1 hypothalamic responses, showing elevated total capacity (AUC) but with normal peak potency, slow rise TSH dynamics with a remarkably delayed peak (at 45, 60 or even 180 minutes) and, similar to H1, very slow rhythm of TSH fall not reaching basal TSH levels at the end of the test. In the model of Dietrich *et al*, TSH-FT4 relation of most patients is located below 2% of pituitary and some of them below 2% of thyroid normal responses, presenting very low levels of FT4 with normal or slightly elevated TSH levels, compatible with severe defects.

Interestingly, all of our patients with H2 responses had CPHD associated with the triad of pituitary and stalk malformations, which is compatible with the van Tijn *et al.* hypothalamic response (type 3), observed in patients who also had CPHD and morphological alterations in the pituitary gland. This type of H2 response deserves a specific conceptual analysis because it could be the cause of the controversies about the ability to discriminate between predominantly pituitary and hypothalamic defects. Mehta *et al.* argued that the etiological discrimination through the TRH test was not very effective, asking: Why should hypothalamic response (hypothalamic failure) be present in patients with CPHD and obvious malformations in the pituitary gland? Why the TRH test is not able to translate into a (expected) pituitary response the so severe and demonstrable disorders in the pituitary gland?

Recall that only our study formally identifies the existence of this two subtypes of hypothalamic TSH response: Mehta *et al* can not identify it by performing a short TRH test (being very important the criterion of non-recovery of basal TSH) and van Tijn *et al*, who is able to evidence some of these curves in their patients assimilates H2 type into a single group of "hypothalamic failure responses" along with the H1 responses of our classification.

The definitive solution of this enigma or of the discordance between "expected" responses and "found" responses in the TRH test of patients with CPHD could be explain taken into account the developmental embryology of pituitary gland and a very particular radiological finding in one of our patients with H2 response.

It is more accepted that the processes of development of the hypothalamus and pituitary are closely related and that the correct formation of adenohypophysis, neurohypophysis and stalk

is due to morphogenic stimuli coming from the hypothalamus (Mehta A *et al.*, 2003). Therefore, it would not be strange that the malformations frequently described as pituitary (forming the triad syndrome) actually correspond to malformations of the hypothalamic-pituitary development. However, the existence of possible hypothalamic malformations, including hypoplasia, is not frequent, probably because there is not much experience in the definition of the borders that delimit the hypothalamic area to allow comparisons of sizes in a homogeneous way, in the absence of consensual references on their size in different ages and sex of the pediatric age. Recently, a methodology for the comparative study of hypothalamic size and volume by special MRI techniques has been described, based on the definition of the anatomical borders of the hypothalamus established by Gabery et al (Follin C *et al.*, 2016; Gabery S *et al.*, 2014). A modified method has allowed the diagnosis in patient # 24 of hypothalamic and pituitary hypoplasia, with respect to controls of their age and sex. This finding suggests that, as in this case, other patients with H2 response may also have hypothalamic (non-diagnosed) malformations associated with pituitary malformations, more evident to classic MRI than, jointly, are responsible for CPHD that all our H2 patients presented. Detailed MRI studies are underway in this regard to test whether hypothalamic size could be affected in these patients with H2 response. If confirmed, the enigma of the existence of hypothalamic responses in cases with pituitary (and stalk) malformations could be clarified.

Therefore, we propose a new classification for those patients who have been classically included in the hypothalamic group, but who have pituitary defects studied by magnetic resonance and hormonal profile. Our H2 response would actually be a "mixed" hypothalamic-pituitary response with sufficient functional ability of the thyrotrophic cells to secrete TSH under exogenous TRH stimulus, although with a very aberrant pattern.

*Genetic findings*

The genetics of central hypothyroidism is largely unknown. For many years, mutations in only two genes expressed in the pituitary, *TSHB* and *TRHR* were known to cause the disorder (Dacou-Voutetakis C *et al.*, 1990; Collu R *et al.*, 1997; Bonomi M *et al.*, 2001). Recently, two additional genes, *IGSF1* and *TBLX1*, were added to the list of them very recently described, consistently linked to CCH when they are defective. Both novel genes were recently shown or proposed to regulate expression of the TRHR and the *TSHB* genes, respectively (García M *et al.*, 2017A, Chapter II of this Thesis). Those findings show that CCH has different modes of mendelian inheritance: recessive (*TSHB, TRHR*) or X-linked with variable penetrance (*IGSF1*, *TBLX1*). In chapter IV of this thesis we propose that specifc *TRHR* mutations may be also

inherited in a dominant fashion with a phenotype of mild hyperthyrotropinemia in heterozygotes (amenable for differential diagnosis with mild primary hypothyropidism). With the exception of *TSHB*, mutations in these genes are represented in the repertoire of genetic defects identified in our cohort (TRHR in #25; IGSF1 in #1, #30, #31, #32; TBLX1 in #2 and #33), showing an adequate degree of suspicion for CCH from participating clinicians.

Even when animal models of hypothalamic hypothyroidism exist, human mutations in *TRH* or other relevant genes involved in physiology (transcription, postranslational modifications), of the TRH-tripeptide are so far unknown (Yamada M *et al.*, 1997; Cyr NE *et al.*, 2012).

Despite CPHD representing only the 30% of the etiology in our cohort (12 patients), our study also revealed defects in genes associated with pituitary development or differentiation of pituitary specific cell lines, such as *POU1F1* (#3) (Bircan I *et al.*, 2001) or *BMP4* (Personal communication of Dr. Campos-Barros *et al.*, 2017) presenting TSH deficiency.

There is strong experimental evidence that multiple genes and regulatory mechanisms are involved in TRH and TSH physiology (Davis SW *et al.*, 2010; García *et al.*, 2014, Annex I of this Thesis). The recent advent of next-generation sequencing (NGS) anticipates the further discovery of novel genes participating and influencing necessary functions for the correct functioning of the hypothalamic-pituitary-thyroid axis.

Using an own-designed NGS panel including 390 genes reported to participate in thyroidal phenotypes in humans, animal or cell models, we revealed novel candidate genes to explain for human central hypothyroidism. Mutations in these novel candidate genes must be functionally tested for pathogenicity and further deeper analysis of them is warranted.

Among them, some deserve special comment. We identified mutations in two genes encoding DNA-binding nuclear proteins: *ZFHX3* and *RXRG* in #11, #34 and in #12 and #36 (mother and daughter with the disorder), respectively.

***ZFHX3*** (also known as ATBF1) is a pituitary transcription factor necessary for Pit1 gen early activation (Qi Y *et al.*, 2008). However, it seems particularly important for the synthesis of TSHB (Qi Y *et al.*, 2008). Human mutations are not reported in this gene; however, central CH is a plausible phenotype to expect. Therefore, the 2 mutations identified (p.3519_3526del and p.Arg1108Cys) deserve testing in an inactivation effect of the protein or its crucial interactions with other nuclear proteins like Prop1, B-catenin at the Pit-1 promoter, especially in thyrotropes (Qi Y *et al.*, 2008). ***RXRG*** forms functional heterodimers with the T3 nuclear receptor (TR) to control T3-dependent gene transcription. It is present in thyrotropes and

hypothalamus (Brown NS *et al.*, 2000). RXRG human mutations are not described so far, but it is known that bexarotene (RXRG-specific ligand used at high dose for cutaneous T-cell lymphomas) causes suppression of thyrotropin secretion (Sherman SI *et al.*, 1999). Therefore, the p.Leu277Val mutation identified deserves functional testing for putative effects resulting in excessive TSH or TRH transcriptional suppression (Brown NS *et al.*, 2000).

On the other hand, we also identified genetic variants in genes encoding nuclear proteins not directly binding DNA, the so called "mediators" of transcription, them being co-activators or co-repressors. ***NR4A1*** (also termed **Nurr77**) mediates the TRH-induced transcription of *TSHB* and also that of *FSHB* (Nakajima Y *et al.*, 2012). The identification of a mutation in a woman with central hypothyroidism and subfertility, requiring *In vitro* fertilization is relevant and is being functionally tested at the moment of writing this thesis. The major regulatory protein complex of *TSHB* transcription is the NCoR-SMRT complex. It is composed by different, intimately interacting nuclear proteins, including NCOR1, NCOR2, TBL1X and its receptor (TBL1XR1), GPS2, NCOA3 (also termed SRC-3) and NCOA1 (also termed SRC1).

*Phenotype-genotype correlations*

In physio-pathological terms, **P1 response** strongly suggests the existence of severe alterations in the synthesis of TSH, including the transcriptional regulation of TSHB in the thyrotropes. **P2 response** is consistent with pituitary defects combining both synthesis and secretion of TSH.

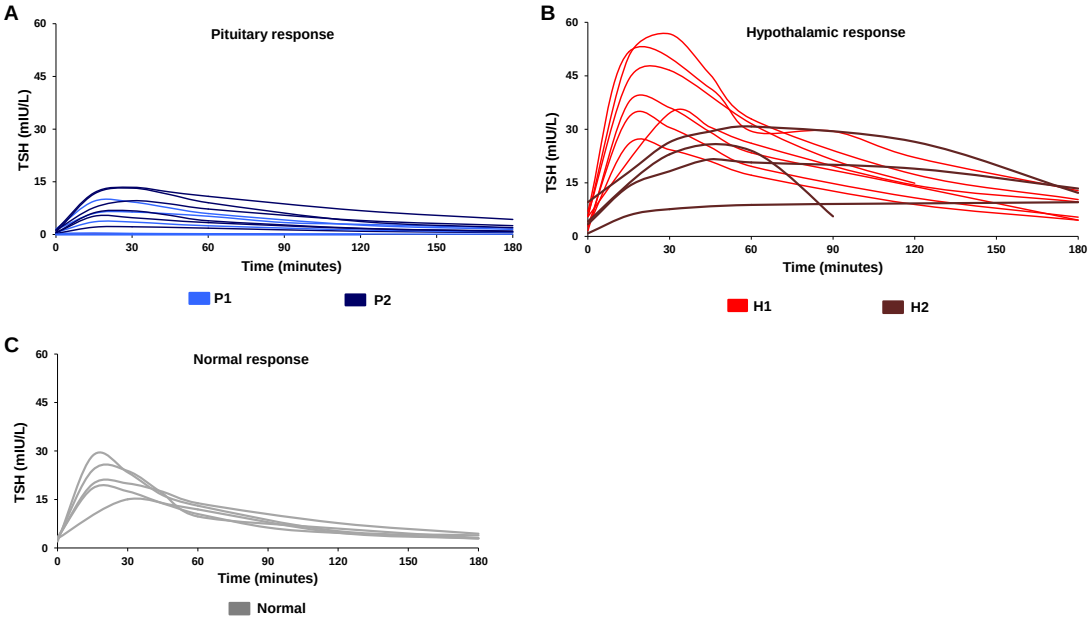
**N response** would signifies that some patients with subtle central hypothyroidism due to mild regulatory or (genetically) mild defects may show an essentially normal TSH secretion and dynamics, subtle enough to not be captured by the test. Confirmation of central hypothyroidism (and not subtle primary thyroidal defects) is advised.

**H1 response** indicates a basic hypothalamic problem of TSH synthesis, secretion (postranslational processing) or arrival (portal circulation, through the stalk) to the pituitary thyrotropes, whose activity is normal. Exaggerated (but timely) secretion of TSH to the bloodstream would explain the slower clearance of very high TSH concentration in the serum.

**H2 response** may indicate the existence of a mixed hypothalamic and pituitary problem with a component of poor synthesis and delayed arrival of TRH to the pituitary thyrotropes, which may also be altered. This would explain the slow (rising and falling) TSH curve dynamics characteristic of this response. Interestingly, from the morphological point of view, all these patients within the group have CPHD associated with the triad of pituitary malformations in the MRI and one of them with pituitary and hypothalamus hypoplasia.

| P | Gene | Variant | State | Inheritance | dbSNP | 1000 genomes frequency | | | ExAC | Path. Predictors | | CL | R |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | All | Eur | Ibs | | CADD | (D/PD/B) | | |
| 1*# | ***IGSF1*** | 207.873Kb deletion | Hz | X-linked | - | - | - | - | - | - | - | 5 | 1 |
| **2** | ***TBL1X*** | c.1015C>T, p.Arg339Ter | Hz | X-linked | - | - | - | - | - | 39 | - | 5 | - |
| **3*** | ***POU1F1*** | c.793C>T, p.Arg265Trp | Hom | Recessive | *rs780359925* | - | - | - | 0% | 34 | 6/0/0 | 5 | 6† |
| **9** | *NCOA1* | c.1528C>A, p.Pro510Thr | Het | - | - | - | - | - | - | 26 | 4/1/1 | 3 | - |
| **11** | *ZFHX3* | c.10557_10577del, p.3519_3526del | Het | - | - | - | - | - | 0% | - | - | 3 | - |
| | *CXCL12* | c.209T>C, p.Val70Ala | Het | - | - | - | - | - | 0% | 26.5 | 4/0/1 | 3 | - |
| **12** | *RXRG* | c.829C>G, p.Leu277Val | Het | S. dominant | - | - | - | - | - | 26.6 | 5/1/0 | 3 | - |
| **18** | *TG* | c.7642G>T, p.Gly2548Cys | Het | - | - | - | - | - | - | 25 | 5/1/0 | 3 | - |
| | *GPS2* | c.607G>A, p.Ala203Thr | Het | - | - | - | - | - | 0% | 21 | 2/0/4 | 3 | - |
| **21** | *PDE4A* | c.274C>T, p.Pro92Ser | Het | - | *rs150660796* | 0.3% | 0% | 0% | 0.3% | 19.2 | 3/3/0 | 3 | - |
| | *TSHB* | c.59C>G, p.Ser20Cys | Het | - | - | - | - | - | 0% | 22.9 | 5/1/0 | 3 | - |
| **25** | ***TRHR*** | c.392T>C, p.Ile131Thr | Hom | Recessive | - | - | - | - | 0% | 23.5 | 5/0/1 | 5 | 2 |
| **26** | *NCOR1* | c.2182+1G>T | Het | *De novo* | - | - | - | - | - | 26.8 | - | 4 | - |
| **28** | *NCOA3* | c.1114G>A, p.Glu372Lys | Het | - | - | - | - | - | - | 27.6 | 5/0/1 | 3 | - |
| **30*** | ***IGSF1*** | c.3488-59_3775delinsCAATAAG | Hz | X-linked | - | - | - | - | - | - | - | 5 | - |
| **31*** | ***IGSF1*** | c.3488-59_3775delinsCAATAAG | Hz | X-linked | - | - | - | - | - | - | - | 5 | - |
| **32#** | ***IGSF1*** | 1727.399Kb deletion | Hz | X-linked | - | - | - | - | - | - | - | 5 | - |
| **33#** | ***TBLX1*** | 9.6Mb deletion (Xp22.33-p22.2) | Hz | X-linked | - | - | - | - | - | - | - | 5 | - |
| **34** | *ZFHX3* | c.3322C>T, p.Arg1108Cys | Het | - | *rs771775824* | - | - | - | 0% | 28.2 | 5/1/0 | 3 | - |
| **36*** | *RXRG* | c.829C>G, p.Leu277Val | Het | S. dominant | - | - | - | - | - | 26.6 | 5/1/0 | 3 | - |
| **49*** | *NR4A1* | c.1688G>A, p.Arg563Gln | Het | - | *rs144165581* | 0.04% | 0% | 0% | 0.03% | 34 | 5/1/0 | 3 | - |

**Table 1: Genetic findings in patients with central congenital hypothyroidism. P**, Patient studied. Underlined: patienst with combined pituitary hormone deficiency (CPHD); **Gene**, Genes **in bold** are classically described in patients with *isolated* central congenital hypothyroidism (CCH) or in CPDH; * Patients genetically investigated by PCR and Sanger sequencing on candidate genes (see Methods), # Patients studied with Comparative Genomic Hybridization (CGH)-array. State, carrier state of the patient: Hz, hemizygous; Het, heterozygous, Hom, homozygous; **Inheritance**: S. dominant, suggested dominant; Freq: population frequency, All: population worldwide; Eur: Europeans; Ibs, Iberians, **Path. Predictors**: *in silico* predictors of pathogenicity (CADD, Sift, Polyphen2, MutAssesor, Fathmm, VEST), number of programs resulting, respectively, in: D, damaging, PD, probably damaging, B, benign; **CL**: functional classification of variants (1, benign; 2, probably benign; 3: uncertain significance, 4: probably pathogenic, 5: pathogenic). R: bibliographic reference, † Described in CPHD in the human gene mutation database (HGMD).




**D**

| | | Secretion capacity | | | | | Secretion dynamics | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **AUC** | **Basal TSH** | **Peak TSH** | **TSH 180'** | **TSH peak/0'** | **Peak time** | **TSH 15'/0'** | **TSH 30'/0'** | **TSH 30'/60'** | **TSH 30'/180'** | **TSH 180'/0'** | |
| | | *1385 *(± 452)* | 0.5-4.7 mIU/L | >15 mIU/L | 0.5-4.7 mIU/L | 6.06 *(± 4.05)* | 15-30 Min | 4.53 *(± 2.71)* | 6.06 *(± 4.05)* | 1.32 *(± 0.15)* | 4.76 *(± 0.96)* | 1.25 *(± 0.64)* | **Type 0** (van Tijn) |
| **Pituitary** | **P1** *N= 6* | **422** *(±441)* | 0.64 *(± 0.59)* | **3.35** *(± 3.57)* | 0.70 *(± 0.72)* | **3.65** *(± 2.18)* | 21 *(± 7.35)* | **3.87** *(± 2.27)* | **3.54** *(± 2.21)* | 1.27 *(± 0.21)* | 3.59 *(± 1.75)* | 1.00 *(± 0.37)* | *Mean (S.D)* |
| | | **253** | **0.44** | **2.02** | **0.42** | **3.57** | 15 | 4.62 | **3.56** | 1.32 | 3.70 | 1.05 | *Median* |
| | **P2** *N = 6* | **1097** *(±594)* | 0.73 *(± 0.38)* | **8.40** *(± 4.11)* | 1.85 *(± 1.28)* | **12.11** *(± 4.77)* | 27.50 *(± 5.59)* | **10.93** *(± 4.21)* | **11.96** *(± 4.81)* | 1.40 *(± 0.17)* | 5.12 *(± 1.75)* | **2.38** *(± 0.63)* | *Mean (S.D)* |
| | | **1025** | **0.55** | **8.17** | 1.46 | **10.86** | 30 | **10.24** | **10.42** | 1.37 | 4.44 | **2.05** | *Median* |
| **Hypo-thalamic** | **H1** *N = 7* | **4677** *(±1344)* | 4.46 *(± 1.87)* | **40.93** *(± 10.22)* | **8.88** *(± 3.96)* | **10.92** *(± 5.50)* | 21.43 *(± 7.42)* | **11.32** *(± 5.72)* | **10.96** *(± 5.47)* | 1.49 *(± 0.12)* | 5.60 *(± 1.38)* | **1.97** *(± 0.41)* | *Mean (S.D)* |
| | | **4530** | **5.52** | **37.62** | **9.65** | **9.82** | 15 | **9.09** | **9.30** | 1.49 | 5.50 | **1.81** | *Median* |
| | **H2** *N = 4* | **3643** *(±1642)* | 4.51 *(± 3.19)* | **21.49** *(± 7.67)* | **10.19** *(± 2.99)* | 6.68 *(± 2.97)* | **86.25** *(± 54.47)* | 4.35 *(± 2.09)* | 5.67 *(± 2.20)* | **0.89** *(± 0.04)* | **2.12** *(± 1.26)* | **5.55** *(± 4.27)* | *Mean (S.D)* |
| | | **3255** | 3.79 | **22.8** | **10.89** | 6.05 | **60** | 4.14 | 5.44 | **0.87** | **1.78** | **3.96** | *Median* |
| **Normal** *N = 5* | | 1781 *(±192)* | 3.04 *(± 0.16)* | 21.21 *(± 4.66)* | 3.45 *(± 0.60)* | 6.41 *(± 1.20)* | 21 *(± 7.35)* | 6.80 *(± 1.11)* | 6.32 *(± 1.22)* | 1.72 *(± 0.39)* | 5.12 *(± 0.80)* | 1.10 *(± 0.21)* | *Mean (S.D)* |
| | | 1800 | 2.98 | 19.96 | 3.08 | 6.09 | 15 | 6.50 | 5.95 | 1.47 | 5.10 | 1.04 | *Median* |

**Figure 1: TSH secretion dynamics at the TRH test in patients with Central Congenital Hypothyroidism.** Graphical representation of serum TSH curves in response to TRH stimulation classified in: **A:** pituitary responses, **B**: , hypothalamic responses, **C:** normal responses, following parameters used by van Tijn *et al. and* represented in the table (**D**): TSH-to-TRH responses were divided in 3 basic groups: pituitary, hypothalamic and normal. Pituitary responses could be further divided in two homogeneous groups: pituitary 1 (P1) and pituitary 2 (P2). Hypothalamic responses were divided also in two groups: hypothalamic 1 (H1) and hypothalamic 2 (H2), based on the following calculations of nude data from the TRH test: AUC, area under the curve (representing the *total capacity* of TSH secretion), Peak TSH (representing the *absolute maximal capacity -or potency-*), TSH peak/0´ (the *relative maximal capacity*) and the dynamics of TSH secretion throughout the test (investigated by the TSH 15'/0', 30'/0', 30'/60'. 30'/180' and 180'/0' ratios). In blue and red are values below or above the mean values of TSH *type 0 response (Normal)* from van Tijn *et al*, respectively (van Tijn DA *et al.*, 2008B). * AUC reference values are taken from 95 controls (unpublished data).

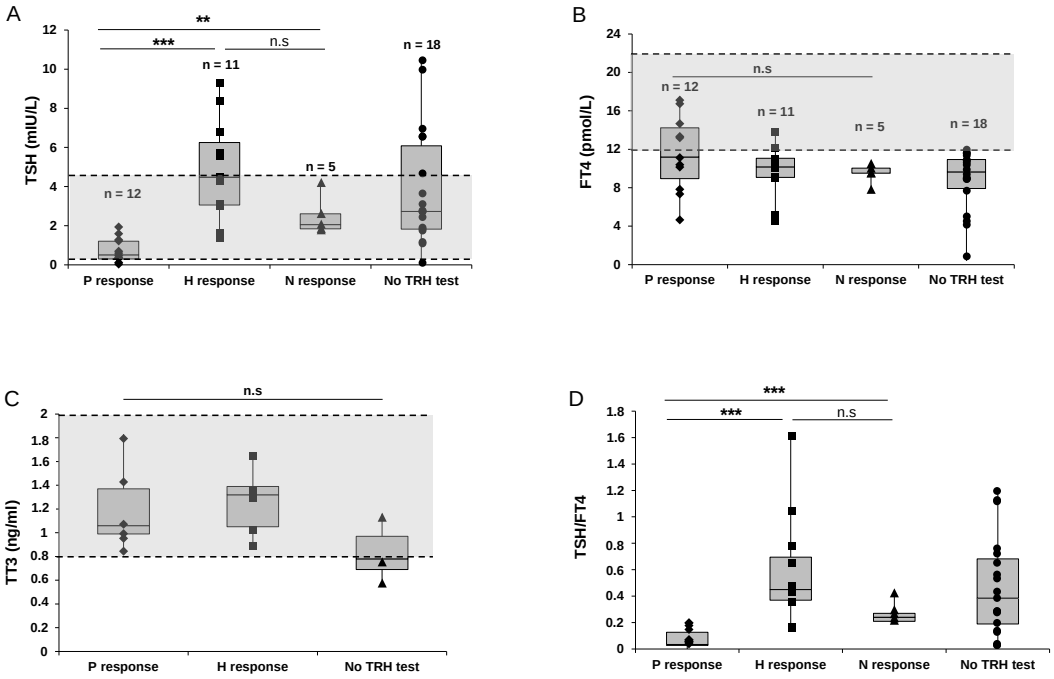



**Figure 2: Hormone profile (TSH, FT4, TT3 and TSH/FT4 ratio) of groups of central hypothyroid (CCH) patients as classified according to their TSH response curves at the TRH test. A**, Individual and mean serum TSH concentrations of patients with pituitary (P), hypothalamic (H) and normal (N) responses and in CCH patients who did not undergo the TRH test, showing significant differences between pituitary and hypothalamic and between pituitary and normal responses, respectively. **B**, Free T4 (FT4) concentrations showing no significant differences between groups. **C**, Total T3 (TT3) concentrations in patients with P and H responses and in patients without TRH test, showing no significant differences between groups. **D**, TSH/FT4 ratio showing significant differences between patients with P and H responses and between P and N responses Grey shadowed area represents normal interval for each paramenter (*p < 0.05, **p < 0.01, ***p < 0.005, n.s ,non significant).

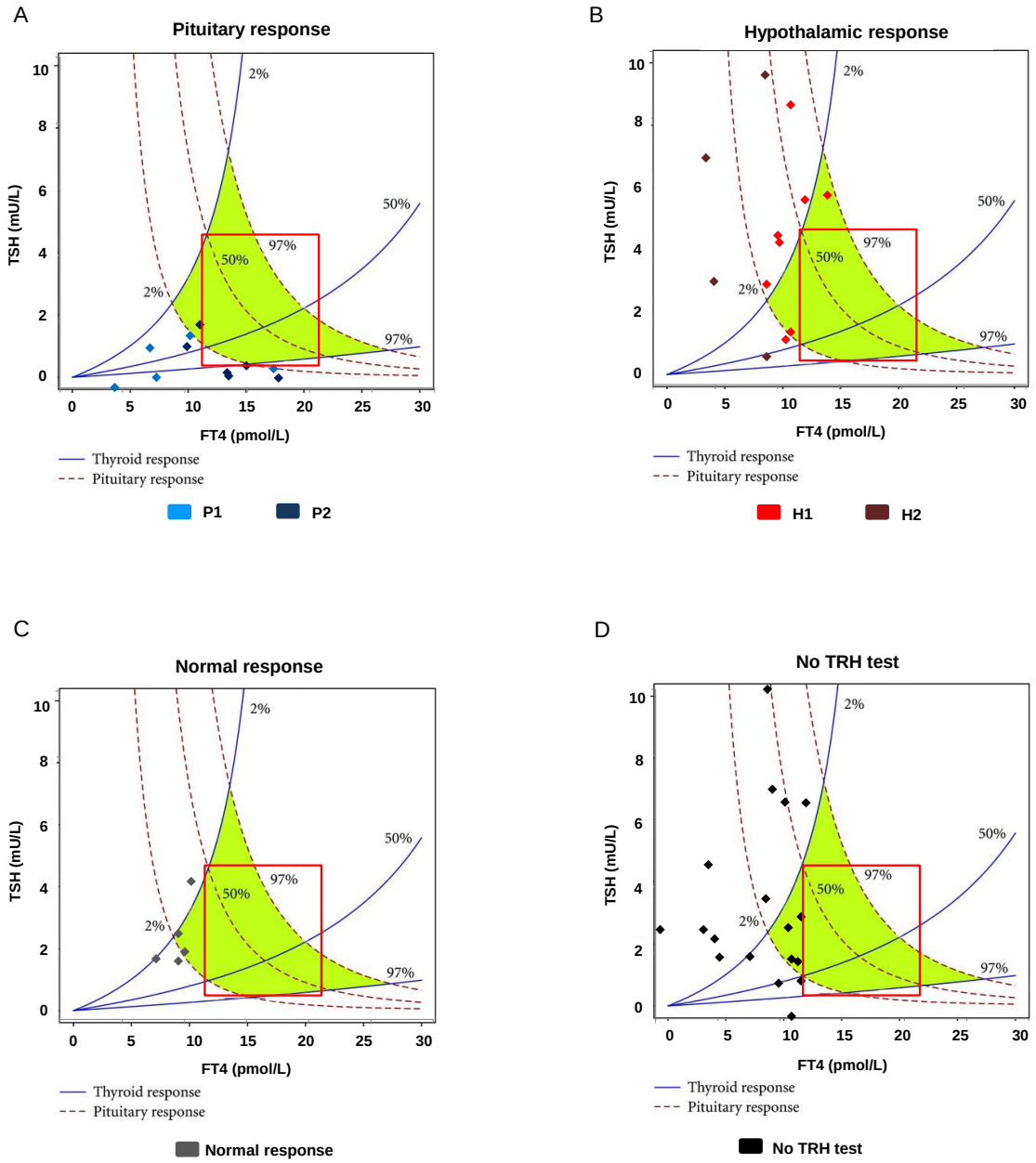



**Figure 3: TSH and FT4 concentrations of patients classified upon their TSH responses to TRH plotted in the dynamic model graph from *Dietrich et al.*** (Dietrich JW *et al*., 2012). The model is based on the comparison of conventional univariate reference ranges for TSH and FT4 (red square) and a bihormonal reference region (green kite-like area) constructed from nonlinear modeling of thyroid homeostasis. Represented in red dotted lines are the 2%, 50% and 97% centiles of the pituitary TSH secretory capacity through a complete range of serum thyroid hormone concentrations. In blue continuous lines, the 2%, 50% and 97% centiles of FT4 concentrations expected from the thyroid gland to secrete in response to a complete range of serum TSH concentrations secreted by the pituitary. **A**, TSH-FT4 plots of patients with pituitary responses at the TRH test. All dots are located outside the red square and green area and, with 1 exception, all locate below the 2$^{nd}$ centile of normal pituitary performance **B,** TSH-FT4 relation of patients with hypothalamic response. Most of patients are located below 2% of the normal thyroid response (consistent with low TSH bioactivity derived from failure of hypothalamic TRH signaling), with the exception of three patients overlapping with pituitary defects. **C,** TSH-FT4 relation in patients (grey dots) with normal TSH curve at the TRH test located in a grey area, overlapping with pituitary and hypothalamic response. **D**, TSH-FT4 relation of patients without TRH test distributed throughout the graph.

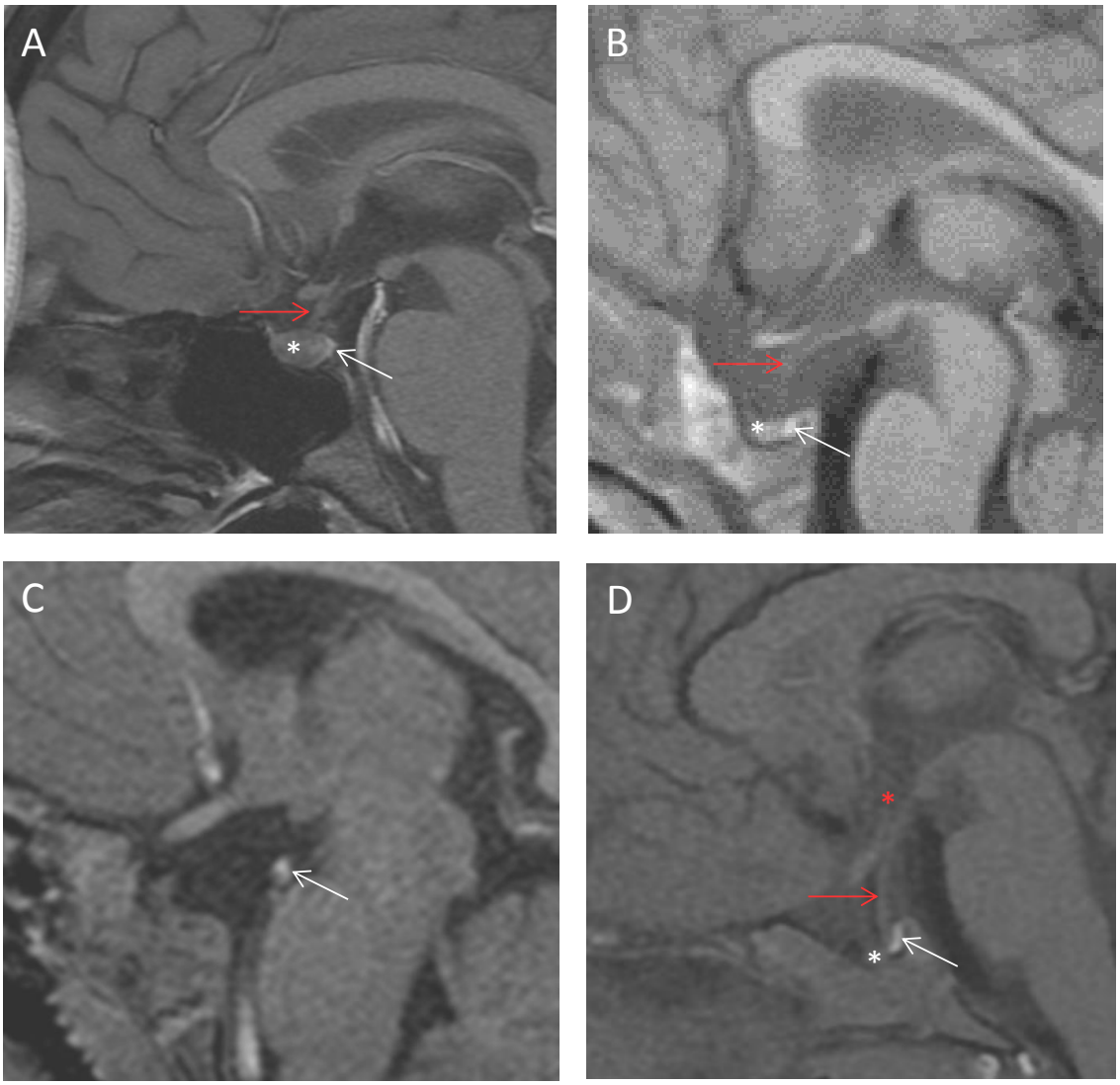



**Figure 4: Magnetic resonance imaging (MRI) of the brain at the pituitary, stalk and hypothalamic area in representative patients of our CCH cohort.** (A) Normal morphology, structure and size of pituitary and stalk. (B) Hypoplastic adenohypophysis, eutopic neurohypophysis and conserved pituitary stalk. (C) Hypoplastic adenohypophysis, neurohypophysis ectopia and absence of pituitary stalk (the TRIAD sign) (D) Adenohypophysis and hypothalamus hypoplasia with eutopic neurohypophysis and normal stalk. White and red arrows represent pituitary neurohypophysis and stalk, respectively. White and red asterisk represent pituitary adenohypophysis and hypothalamus, respectively.

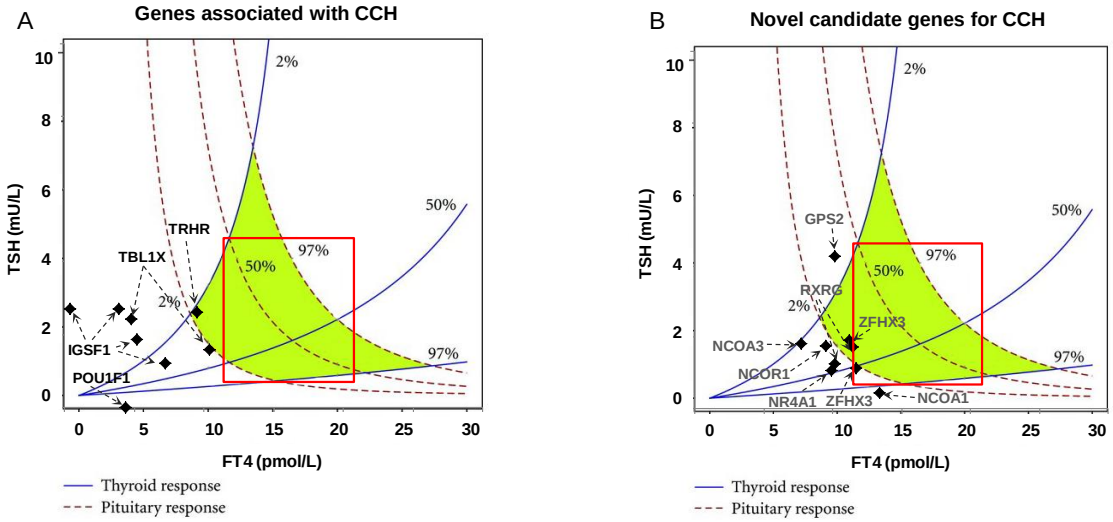



**Figure 5: TSH/FT4 relation at the Dietrich´s model in patients harboring genetic variants identified in this study.** (A) TSH/FT4 relation in patients carrying pathogenic mutations in genes previously associated with CCH of pituitary origin, compatible with their location borderline or below 2% of normal pituitary response (red dotted line) (B) TSH/FT4 relation in patients carrying variants in novel genes proposed as candidates for CCH.

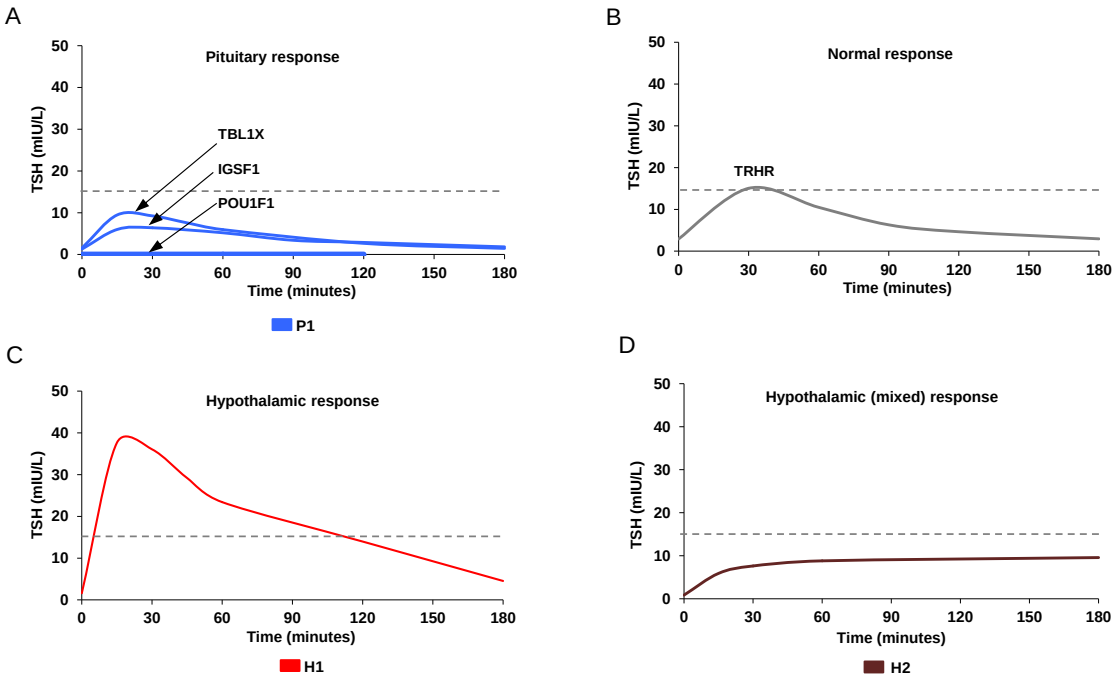



**Figure 6: TSH secretion dynamics in patients with known origin of central hypothyroidism based on the identification of genetic defects or through clinical or radiological studies.** (A) Patients with pathogenic mutations in genes expressed in pituitary causing severe (*POU1F1*) or moderate (*IGSF1*, *TBL1X*) defects in TSH synthesis, showed at the TRH test as pituitary type 1 (P1) response. (B) Patient with pathogenic mutation in *TRHR* causing mild defect on TSH synthesis that is not appreciate at the TRH test, showing normal TSH response under TRH stimulus. (C) Patient with hypothalamic hypothyroidism consistent with hypothalamic type 1 (H1) response at the TRH test associated with hypothalamic hypermetabolism. (D) Patient with pituitary and hypothalamus hypoplasia consistent with mixed response at the TRH test.

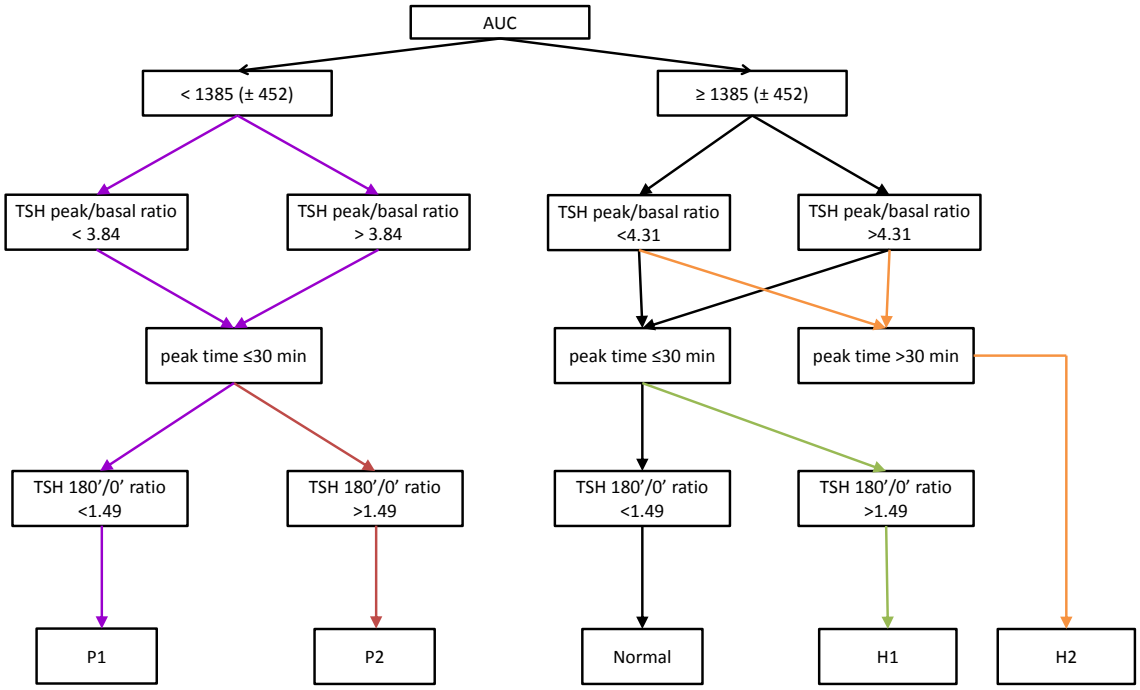



**Figure 7: Decision-making diagram to classified different TSH responses at the TRH test, according to the data reported in this study.** Reference values for AUC are taken from 95 control individuals (not published data of our laboratory). Reference values for TSH peak/basal ratio of pituitary response (< or > 3.84), and normal and hypothalamic responses (< or >4.31) represent maximum 30'/0' ratio of type 2 response (mean + SD) and minimum of 30'/0' ratio of type 3 response (mean – SD), respectively, according to van Tijn *et al*. (van Tijn DA *et al.*, 2008B). TSH 180'/0' reference ratio was obtained from maximum ratio of type 2 responses of van Tijn *et al.* (mean + SD) (van Tijn DA *et al.*, 2008B). P1: pituitary type 1 response, P2: pituitary type 2 response, H1: hypothalamic type 1 response, H2: hypothalamic type 2 response.

| P | TSH resp | Age Y | Sex | Clinical features | TSH mU/L 0.5-4.7 | FT4 pmol/L 11.6-21.9 | TT3 ng/ml 0.8-2 | TSH/FT4 0.027-0.13 | MRI | Genes |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | P1 | 0.04 | M | Macroorchidsm | 1.2 | **7.21** | | **0.17** | N | *IGSF1* |
| 2 | P1 | 6 | M | ADHD<br>Hearing loss | 1.57 | **10.42** | 1.46 | **0.15** | N<br>Arnold-Chiari I | *TBL1X* |
| 3 | P1 | 0.01 | F | See CPHD table (Supp 3) | **<0.005** | **4.38** | - | **0.00** | N | *POU1F1* |
| 4 | P1 | 2 | F | Cardiopathy and coarse facies | **0.31** | **7.72** | - | 0.04 | N | - |
| 5 | P1 | 43 | F | Cardiopathy and thyroid hypoplasia | **0.17** (LT4) | 20.21 (LT4) | - | **0.01** | - | - |
| 6 | P1 | 60 | F | - | 0.57 | 17.12 | - | 0.03 | N | - |
| 7 | P2 | 12 | F | Short stature<br>See CPHD table (Supp 3) | 0.67 | 14.93 | 0.98 | 0.04 | Hypoplastic AP | - |
| 8 | P2 | ? | M | - | **0.3** | 17.50 | - | **0.02** | - | - |
| 9 | P2 | 10 | F | Short stature | **0.46** | 13.38 | 1.1 | 0.03 | - | *NCOA1* |
| 10 | P2 | 13 | F | Autism and epilepsy | **0.36** | 13.51 | 0.87 | 0.03 | N | - |
| 11 | P2 | 7 | M | - | 1.91 | **11.20** | 1.83 | **0.17** | - | *ZFHX3*<br>*CXCL12* |
| 12 | P2 | 59 | F | - | 1.25 | **10.17** | 1.02 | 0.12 | - | *RXRG* |
| 13 | H1 | 12 | M | - | **8.4** | **11.07** | - | **0.76** | - | - |
| 14 | H1 | 40 | M | - | **4.48** | **10.04** | 0.91 | **0.45** | N | - |
| 15 | H1 | 1.5 | F | Growth delay | 1.58 | **11.07** | 1.32 | **0.14** | Hypoplastic AP | - |
| 16 | H1 | 28 | F | Subfertility | 1.35 | **10.68** | - | 0.13 | - | - |
| 17 | H1 | 12 | F | Short stature and obesity | **5.55** | 12.23 | 1.68 | **0.45** | - | - |
| 18 | H1 | 3 | M | Psychomotor retardation and language delay | 4.27 | **10.17** | - | **0.42** | N | *TG*<br>*GPS2* |
| 19 | H1 | 10 | F | Shapiro S. | 3.01 | **9.14** | 1.05 | **0.33** | Hypoplastic AP | - |
| 20 | H1 | 13 | M | Shapiro S. | **5.69** | 14.03 | 1.39 | **0.41** | N | - |
| 21 | H2 | 15 | M | Mental retardation<br>See CPHD table (Supp 3) | 3.1 | **4.89** | - | **0.63** | Hypoplastic AP<br>Stalk absent<br>Ectopic PP<br>Arnold-Chiari I | *TSHB*<br>*PDE4A* |
| 22 | H2 | 24 | F | See CPHD table (Supp 3) | **6.81** | **4.25** | - | **1.60** | Hypoplastic AP<br>Stalk absent<br>Ectopic PP | - |
| 23 | H2 | 12 | F | Insulin resistance<br>See CPHD table (Supp 3) | **9.3** | **9.01** | - | **1.03** | Hypoplastic AP<br>Ectopic PP | - |
| 24 | H2 | 3 | F | See CPHD table (Supp 3) | 0.84 | **9.14** | - | 0.09 | Hypolastic AP and hypothalamus | - |
| 25 | N | 8 | M | Obesity | 2.61 | **9.52** | - | **0.27** | N | *TRHR* |
| 26 | N | 4 | F | Hyperactivity and language difficulties | 1.77 | **9.52** | - | **0.19** | - | *NCOR1* |
| 27 | N | 12 | M | Psychomotor retardation, corneal dystrophy, keratopathy | 2.06 | **10.04** | - | **0.21** | N | - |
| 28 | N | 0.25 | F | congenital cardiopathy | 1.84 | **7.72** | - | **0.24** | N | *NCOA3* |
| 29 | N | 16 | M | See CPHD table (Supp 3) | 4.2 | **10.55** | - | **0.40** | N | |

**Supplemental Table 1: Detailed clinical and genetic features of patients with TRH test.**

P: patient number, TSH resp: Type of TSH response, P1: pituitary type 1, P2: pituitary type 2, H1: hypothalamic type 1, H2: hypothalamic type 2, N: normal, Y: years, ?: unknown, M: male, F: female, S.: syndrome, ADHD: attention deficit-hyperactivity disorder, CPHD: combined pituitary hormone deficiency, Supp 3: Supplemental Table 3, L-T4: under Levo-thyroxine treatment, MRI: magnetic resonance image, AP: anterior pituitary, PP: posterior pituitary. - : not available. In blue and red are represented values below and above normal reference ranges, respectively.

| P | Age Y | Sex | Clinical features | TSH mU/L 0.5-4.7 | FT4 pmol/L 11.6-21.9 | TT3 ng/ml 0.8-2 | TSH/FT4 0.027-0.13 | MRI | Gene |
|---|---|---|---|---|---|---|---|---|---|
| 30 | 0.01 | M | Macroorchidsm and thyroid hypoplasia | 2.7 | **4** | - | **0.70** | - | *IGSF1* |
| 31 | 0.01 | M | Macroorchidsm and thyroid agenesia | 2.7 | **0.44** | - | **6.99** | - | *IGSF1* |
| 32 | 4 | M | Macroorchidism | 1.86 | **5.15** | - | **0.36** | - | *IGSF1* |
| 33 | 30 | M | Polymalformative S. See CPHD table (Supp 3) | 2.42 | **4.76** | - | **0.51** | - | *TBL1X del* |
| 34 | 26 | F | Rokitansky S. | 1.14 | 11.71 | - | 0.10 | N | *ZFHX3* |
| 35 | 38 | F | - | **0.07** | **10.94** | 1.16 | **0.01** | N | - |
| 36 | 29 | F | Hyperinsulinism and hyperandrogenism | 1.73 | **11.45** | - | **0.15** | - | *RXRG* |
| 37 | 23 | F | Alstrom S. | 3.64 | **8.88** | - | **0.41** | N | - |
| 38 | 10 | M | Epilepsy | **6.96** | **9.40** | - | **0.74** | - | - |
| 39 | 3 | M | Obesity Developmental disorder | 2.76 | **10.68** | - | **0.26** | - | - |
| 40 | 2 | M | - | **6.57** | **10.42** | - | **0.63** | - | - |
| 41 | 0.03 | F | Neonatal hyperthyrotropinemia See CPHD table (Supp 3) | **10.48** | **8.88** | - | **1.18** | Hypoplastic AP Stalk absent Ectopic PP | - |
| 42 | 28 | M | 48, XXYY | 3.09 | 11.71 | - | **0.26** | - | - |
| 43 | 13 | F | Fasting hyperglycemia | **6.55** | 12.10 | N | **0.54** | - | - |
| 44 | 1 | M | Low growth, obesity, macrocephaly See CPHD table (Supp 3) | - | - | - | - | - | *TSHR* |
| 45 | 0.58 | M | See CPHD table (Supp 3) | **10** | **9.01** | - | **1.11** | - | - |
| 46 | 18 | M | See CPHD table (Supp 3) | 1.81 | **10.94** | - | **0.17** | Hypoplastic AP Arnold-Chiari I | - |
| 47 | 12 | M | Macroorchidism See CPHD table (Supp 3) | N | **Low** | - | - | N AP Ectopic PP | - |
| 48 | 16 | M | See CPHD table (Supp 3) | 4.67 | **4.25** | - | **1.10** | Hypoplastic AP Stalk absent | - |
| 49 | 42 | F | See CPHD table (Supp 3) | 1.07 | **9.91** | **0.6** | 0.11 | N | *NR4A1* |
| 50 | 1 | F | Dysmorphic features See CPHD table (Supp 3) | 1.88 | **7.59** | **0.78** | **0.25** | Hypoplastic AP | - |

**Supplemental Table 2: Detailed clinical and genetic features of patients without TRH test.**

P: patient number, Y: years, M: male, F: female, S.: syndrome, CPHD: combined pituitary hormone deficiency, Supp 3: Supplemental Table 3, N: normal, MRI: magnetic resonance image, AP: anterior pituitary, PP: posterior pituitary. - : not available. In blue and red are represented values below and above normal reference ranges, respectively.

| P | TSH resp | Age Y | Sex | Pituitary hormone deficiencies | | | | | | TSH mU/L 0.5-4.7 | FT4 pmol/L 11.6-21.9 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | TSH | GH | FSH | LH | ACTH | PRL | | |
| 3 | P1 | 0.01 | F | x | x | | | | x | **<0.005** | **4.38** |
| 7 | P2 | 12 | F | x | x | x | x | | | 0.67 | 14.93 |
| 21 | H2 | 15 | M | x | x | x | x | x | x | 3.1 | **4.89** |
| 22 | H2 | 24 | F | x | x | x | x | x | x | **6.81** | **4.25** |
| 23 | H2 | 12 | F | x | x | x | x | x | | **9.3** | **9.01** |
| 24 | H2 | 3 | F | x | x | | | | | **0.84** | **9.14** |
| 29 | N | 16 | M | x | | x | x | | | 4.2 | **10.55** |
| 33 | No test | 30 | M | x | x | x | x | | | 2.42 | **4.76** |
| 41 | No test | 0.03 | F | x | x | | | x | | **10.48** | **8.88** |
| 44 | No test | 1 | M | x | x | | | | | - | - |
| 45 | No test | 0.58 | M | x | x | x | x | x | | **10** | **9.01** |
| 46 | No test | 18 | M | x | x | x | x | | | 1.81 | **10.94** |
| 47 | No test | 12 | M | x | x | x | x | | | N | **Low** |
| 48 | No test | 16 | M | x | x | | | x | | 4.67 | **4.25** |
| 49 | No test | 42 | F | x | | x | x | | | 1.07 | **9.91** |
| 50 | No test | 1 | F | x | x | | | | | 1.88 | **7.59** |

**Supplemental Table 3: Combined pituitary hormone deficiencies (CPHD) in patients with CCH.**

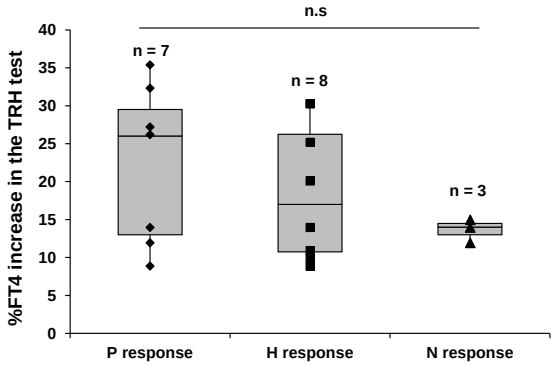
P: patient number, TSH resp: Type of TSH response, P1: pituitary type 1, P2: pituitary type 2, H2: hypothalamic type 2, N: normal, Y: years, M: male, F: female, X: hormone deficiency present. In blue and red are represented values below and above normal reference ranges, respectively.

| P | FT4 Basal | TSH Basal | TSH Peak | TSH 180′ | TSH Peak | TSH 180′ | TSH 15′/0′ | TSH 30′/0′ | TSH 30′/60′ | TSH 30′/180′ | TSH 180′/0′ | FT4 180′/0′ | Quantitative | Dynamics | Type Resp |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| N.R. | 0.9-1.7 mIU/L | 0.5-4.7 mIU/L | >15 mIU/L | 0.5-4.7 mIU/L | 15,30 min | - min | 4.53 (±2.71) | 6.06 (±4.05) | 1.32 (±0.15) | 4.76 (±0.96) | 1.25 (±0.64) | >14% | Normal | Normal | N |
| 1 | - | 1.3 | **6.4** | 1.83 | 30 | 180 | 4.62 | **4.92** | 1.23 | **3.50** | 1.41 | - | Low TSH | Normal | P1 |
| 2 | **0.81** | 1.57 | **9.48** | 1.46 | 15 | 180 | 6.04 | 5.89 | 1.55 | 6.34 | 0.93 | 27 | Low TSH | Normal | P1 |
| 3 | - | **0.005** | **0.005** | 0.005 | - | 120 | - | - | - | - | - | - | Null TSH Null PRL | Null | P1 |
| 4 | **0.6** | **0.31** | **0.43** | 0.08 | 15 | 180 | **1.38** | **1** | 1.4 | **3.9** | 0.25 | - | Low TSH | Normal | P1 |
| 5 | - | **0.09** | **0.2** | 0.1 | 30 | 90 | - | **2.2** | 1 | **2** (30/90) | 1.1 (90/0) | - | Low TSH | Normal | P1 |
| 6 | 1.33 | 0.57 | **3.6** | 0.74 | 15 | 180 | 6.32 | 6.23 | 1.43 | 4.80 | 1.30 | **12** | Low TSH | Normal | P1 |
| 7 | 1.31 | 0.62 | **13.2** | 1.93 | 30 | 180 | **18.60** | **21.29** | 1.46 | **6.84** | **3.11** | 35 | Overstim | Non-recovery | P2 |
| 8 | 1.36 | **0.3** | **2.2** | 0.55 | 30 | 180 | 6.93 | 7.33 | 1.22 | 4.00 | **1.83** | **9** | Low TSH | Non-recovery | P2 |
| 9 | 1.04 | **0.46** | **6.74** | 0.84 | 30 | 180 | **13.41** | **14.65** | 1.72 | **8.02** | **1.83** | 32 | Overstim | Non-recovery | P2 |
| 10 | - | **0.47** | **5.2** | 0.98 | 15 | 180 | **11.06** | **10.17** | 1.41 | 4.88 | **2.09** | - | Overstim | Non-recovery | P2 |
| 11 | **0.91** | 1.26 | **13.43** | 4.29 | 30 | 180 | **9.42** | **10.66** | 1.24 | 3.13 | **3.40** | 26 | Overstim | Non-recovery | P2 |
| 12* | **0.79** | 1.25 | **9.6** | 2.51 | 30 | 180 | 6.13 | 7.68 | 1.32 | 3.82 | **2.01** | 14 | Low TSH | Non-recovery | P2 |
| 13 | **0.82** | **5.52** | **34.57** | **9.65** | 30 | 180 | - | 6.26 | 1.32 | 3.58 | **1.75** | **11** | High TSH | Non-recovery | H1 |
| 14* | **0.73** | **7** | **51.69** | **12.8** | 15 | 180 | **7.38** | - | 1.25 (15/60) | 4.04 (15/180) | **1.83** | **10** | Overstim | Non-recovery | H1 |
| 15 | **0.86** | 1.58 | **37.62** | 4.54 | 15 | 180 | **23.8** | **22.8** | 1.5 | **7.9** | **2.87** | 30 | Overstim | Non-recovery | H1 |
| 16* | **0.83** | 1.35 | **1085** | **332** | 30 | 180 | **796.30** | **803.70** | 1.22 | 3.27 | **245.93** | 17 | Overstim | Non-recovery | H1 |
| 17 | 0.95 | **5.55** | **56.75** | **10.31** | 30 | 180 | **9.08** | **10.23** | 1.72 | 5.50 | **1.86** | 25 | Overstim | Non-recovery | H1 |
| 18 | **0.86** | **5.92** | **46.56** | **14.9** | 30 | 120 | **7.43** | 7.86 | 1.47 | 3.12 (30/120) | **2.86** (120/0) | - | Overstim | Non-recovery | H1 |
| 19 | **0.71** | 3.01 | **33.39** | **5.38** | 15 | 180 | **11.1** | **10.1** | 1.5 | 5.7 | **1.79** | 30 | Overstim | Non-recovery | H1 |
| 20 | 1.22 | 2.64 | **25.92** | 4.55 | 15 | 180 | **9.1** | 8.5 | 1.4 | 5.3 | **1.7** | 20 | Overstim | Non-recovery | H1 |

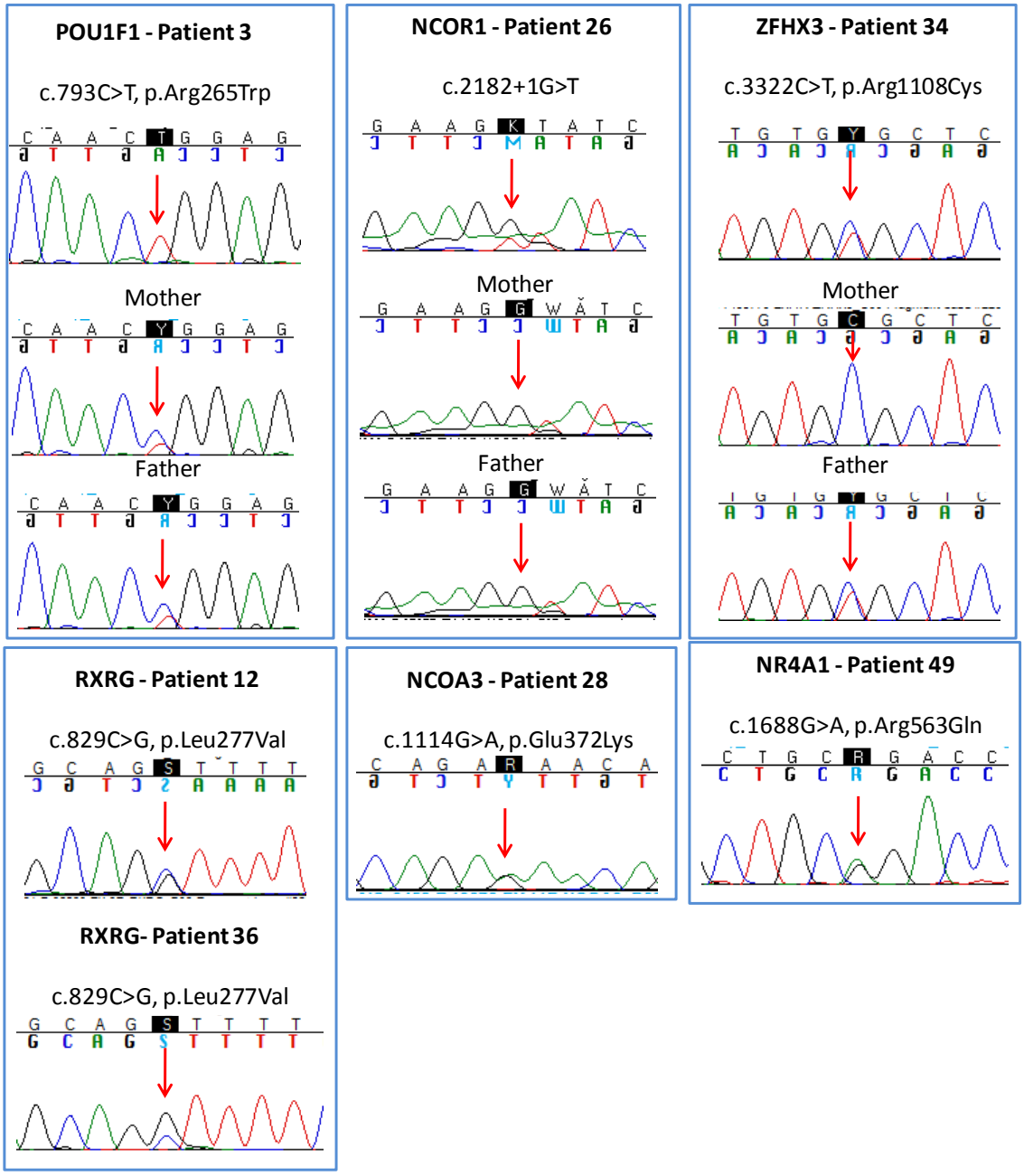
**Supplemental Table 4: Evaluation of secretory capacity and dynamics of TSH at the TRH test in patients with pituitary and hypothalamic responses.** P: patient number, N.R.: normal range, *: adults, min: minutes, Overstim: overstimulation of TSH, Type Resp.: type of response, N: normal, P1: pituitary type 1, P2: pituitary type 2, H1: hypothalamic type 1. In blue and red are represented values below and above reference ranges, respectively. Green and yellow fills represent TSH peak/basal ratio and non-recovery of basal TSH, respectively.

| P | FT4 Basal | TSH Basal | TSH Peak | TSH 180' | TSH Peak | TSH 180' | TSH 15'/0' | TSH 30'/0' | TSH 30'/60' | TSH 30'/180' | TSH 180'/0' | FT4 180'/0' | Quantitative | Dynamics | Type Resp |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| N.R. | 0.9-1.7 mIU/L | 0.5-4.7 mIU/L | >15 mIU/L | 0.5-4.7 mIU/L | 15,30 min | - min | 4.53 (±2.71) | 6.06 (±4.05) | 1.32 (±0.15) | 4.76 (±0.96) | 1.25 (±0.64) | >14% | Normal | Normal | N |
| 21 | **0.35** | 3.38 | 21.6 | 13.4 | 45 | 180 | 4.14 | 5.40 | **0.88** | **1.36** | **3.96** | **9** | Overstim TSH Null PRL | Delayed peak Non-recovery | H2 |
| 22 | - | 4.2 | **24** | 5.6 | 60 | 90 | - | 5.48 | **0.96** | 4.12 (30/90) | 1.33 (90/0) | - | Overstim TSH Null PRL | Delayed peak | H2 |
| 23 | **0.7** | **9.6** | **30.8** | **12.2** | 60 | 180 | **1.9** | **2.8** | **0.85** | **2.2** | 1.3 | 14 | High TSH | Delayed peak | H2 |
| 24 | **0.71** | 0.84 | 9.57 | 9.57 | 180 | 180 | 7 | 9 | **0.86** | **0.79** | **11.4** | 20 | Overstim | Delayed peak Non-recovery | H2 |
| 25 | - | 2.92 | 15.05 | 2.95 | 30 | 180 | - | 5.15 | 1.43 | 5.10 | 1.01 | **12** | Normal TSH | Normal | N |
| 26 | 0.95 | 2.9 | 24.05 | 3.08 | 15 | 180 | **8.29** | 8.21 | **2.45** | 3.98 | 1.06 | 14 | Overstim | Normal | N |
| 27 | - | 3.29 | 18.45 | 2.92 | 15 | 180 | 5.61 | 5.33 | 1.47 | 6.00 | 0.89 | - | Normal TSH | Normal | N |
| 28 | - | - | 28.53 | 3.9 | 15 | 180 | - | - | 1.79 | 6.00 | - | - | - | Normal | N |
| 29 | 1 | 3.04 | 19.96 | 4.4 | 30 | 180 | 6.50 | 6.57 | 1.44 | 4.54 | 1.45 | 15 | Normal TSH | Normal | N |

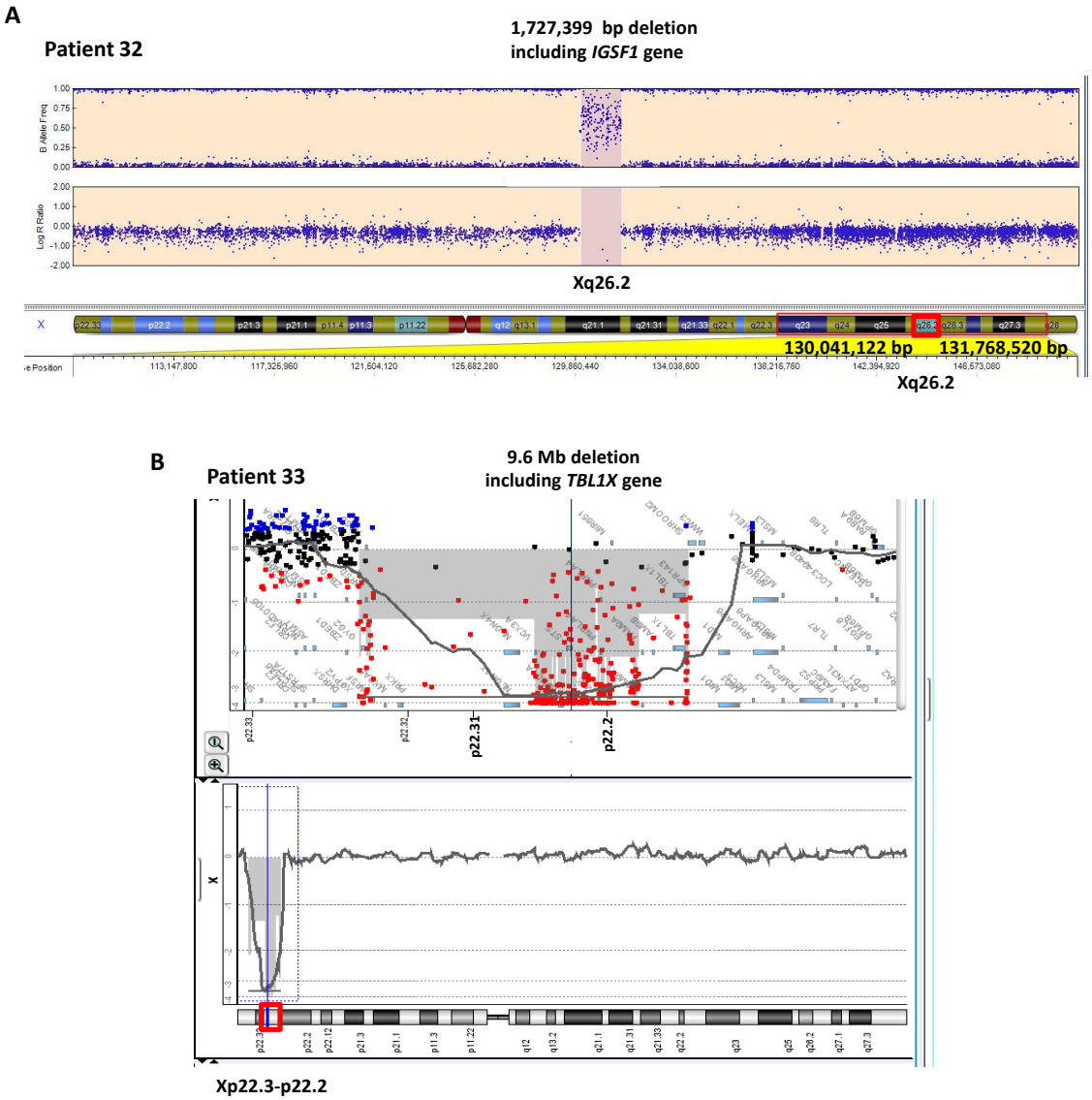
**Supplemental Table 5: Evaluation of secretory capacity and dynamics of TSH at the TRH test in patients with hypothalamic H2 and normal responses.** P: patient number, N.R.: normal range, min: minutes, Overstim: TSH overstimulation, Type Resp.: type of response, H2: hypothalamic type 2, N: normal response. In blue and red are represented values below and above normal reference ranges, respectively. Green fills represents TSH peak/basal ratio.



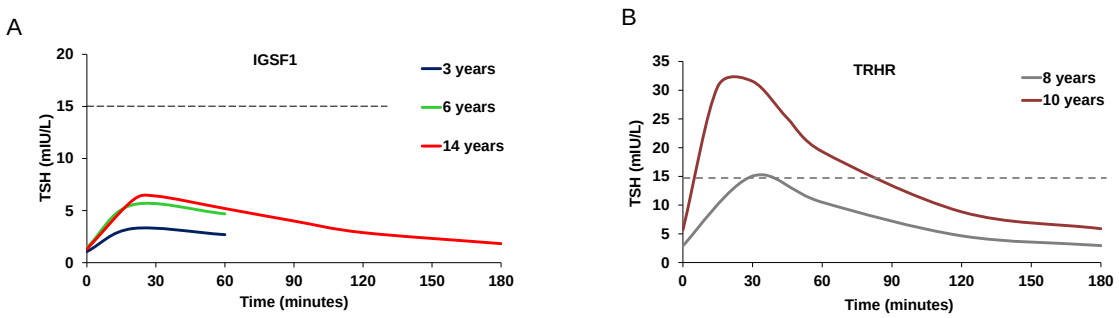

**Supplemental Figure 1: Thyroid hormone response to TRH test: indirect measure of TSH bioactivity.** Percentage of FT4 increase 180 minutes after TRH stimulus showed no significant differences between pituitary (P), hypothalamic (H) and normal (N) responses.




**Supplemental Figure 2: Representative chromatograms of patients with variants identified in known and new candidate genes.**




**Supplemental Figure 3: Comparative Genomic Hybridization (CGH)-Array of two patients with deletion in chromosome X associated with CCH.** (A) CGH-Array (CytoSNP-850K BeadChip) of patient 32 harboring a hemizygous deletion of 1727.399Kb in Xq26.2, completely deleting *IGSF1* gene. (B) CGH-Array (KaryoArray ®v3.0) of patient 33 harboring a hemizygous deletion of 9.6 Mb in Xp22.33-p22.2, completely deleting *TBL1X* gene.




**Supplemental Figure 4: Patients with *IGSF1* (A) and *TRHR* (B) defects were subjected to more than one TRH test, showing an improvement in the TSH peak in response to TRH in each stimulus through age.**

# CAPÍTULO II

## El síndrome de hipotiroidismo central y macroorquidismo: IGSF1 controla la expresión de TRHR y FSHB por modulación diferencial de las vías de TGFβ y Activina en la hipófisis

Los defectos en IGSF1 (factor 1 de la superfamilia de inmunoglobulinas) causan una enfermedad ligada al cromosoma X caracterizada por hipotiroidismo central y macroorquidismo. Sin embargo, los mecanismos patogénicos de la enfermedad son desconocidos. Este estudio está basado en la caracterización fenotípica y genética de un paciente con una deleción completa de *IGSF1* clínicamente seguido desde recién nacido a la edad adulta. Además, se ha investigado la posibilidad de un origen hipofisario común para el hipotiroidismo y el macroorquidismo, así como el papel de *IGSF1* como regulador de la secreción hormonal hipofisaria. El paciente mostró un hipotiroidismo congénito central con reducción de labioactividad de la TSH, una hipersecreción de FSH durante la mini-pubertad neonataly también en la infancia en un test de estímulo con GnRH. Además, a partir de los 3 años de edad desarrolló un macroorquidismo progresivo hasta la edad adulta, con una secreción aumentada de inhibina B que era incapaz de inhibir la secreción de FSH, indicando un estado de resistencia a inhibina B en la hipófisis.

En este estudio se muestra la expresión de IGSF1 tanto en células tirotropas como gonadotropas de la hipófisis y también en células de Leydig y células germinales del testículo, pero con una expresión muy reducida en células de Sertoli. Además, IGSF1 estimula la transcripción del receptor de la hormona liberadora de tirotropina (TRHR) a través de la modulación negativa de la vía de señalización TGFβ1-Smad, y favoreciendo de forma indirecta la síntesis y biopotencia de la TSH, hormona secretada en células tirotropas. Por el contrario, IGSF1 regula negativamente la vía de señalización Activina-Smad, lo que conduce a una reducción en la expresión de FSHB, hormona secretada por células gonadotropas. El mecanismo de actuación de IGSF1 en la hipófisis podría explicar cómo defectos en este gen producen una menor expresión de TRHR y una estimulación anormalmente elevada de la síntesis de FSH, conduciendo al hipotiroidismo central y al macroorquidismo. En conclusión, se han identificado dos mecanismos moleculares relevantes relacionados con el hipotiroidismo central y el macroorquidismo producidos por defectos en IGSF1, revelando a IGSF1 como un importante regulador de las vías de TGFβ y Activina en la hipófisis.

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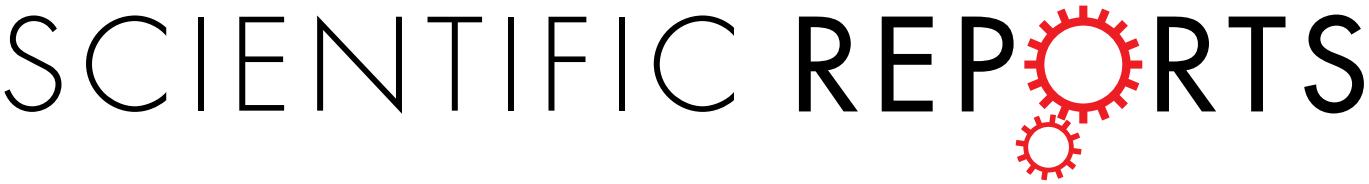

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# The syndrome of central hypothyroidism and macroorchidism: IGSF1 controls *TRHR* and *FSHB* expression by differential modulation of pituitary TGFβ and Activin pathways

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***IGSF1*** **(*Immunoglobulin Superfamily* 1) gene defects cause central hypothyroidism and macroorchidism. However, the pathogenic mechanisms of the disease remain unclear. Based on a patient with a full deletion of *IGSF1* clinically followed from neonate to adulthood, we investigated a common pituitary origin for hypothyroidism and macroorchidism, and the role of IGSF1 as regulator of pituitary hormone secretion. The patient showed congenital central hypothyroidism with reduced TSH biopotency, over-secretion of FSH at neonatal minipuberty and macroorchidism from 3 years of age. His markedly elevated inhibin B was unable to inhibit FSH secretion, indicating a status of pituitary inhibin B resistance. We show here that IGSF1 is expressed both in thyrotropes and gonadotropes of the pituitary and in Leydig and germ cells in the testes, but at very low levels in Sertoli cells. Furthermore, IGSF1 stimulates transcription of the thyrotropin-releasing hormone receptor (*TRHR*) by negative modulation of the TGFβ1-Smad signaling pathway, and enhances the synthesis and biopotency of TSH, the hormone secreted by thyrotropes. By contrast, IGSF1 strongly down-regulates the activin-Smad pathway, leading to reduced expression of FSHB, the hormone secreted by gonadotropes. In conclusion, two relevant molecular mechanisms linked to central hypothyroidism and macroorchidism in IGSF1 deficiency are identified, revealing IGSF1 as an important regulator of TGFβ/Activin pathways in the pituitary.**

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Central Congenital Hypothyroidism (CCH) is a group of hypothalamic-pituitary disorders leading to deficient thyrotropin (TSH) secretion and low thyroid hormone (T4 and T3) synthesis from an otherwise normal thyroid gland[1,2]. CCH may coexist with the failure of other pituitary hormones but is rarely associated with non-hormonal clinical features. In 2009, we described the clinical association of familial isolated central hypothyroidism and testicular enlargement, suggesting a genetic nature of the disorder[3]. Recently, such combined (hypophyseal-testicular) phenotype was linked to defects in the *IGSF1* gene in male adolescents and adults[4]. Although, not all IGSF1 deficient patients present macroorchidism[5].

*IGSF1* gene is located in chromosome Xq26 and encodes a member of the *Immunoglobulin Superfamily* of membrane proteins[6]. IGSF1 contains twelve C2-type immunoglobulin (Ig) loops, a transmembrane domain and a short intracellular C-terminal tail. Despite the presence of Ig loops in its structure, IGSF1 is devoid of independent tyrosine kinase activity[7]. Its function and molecular mechanisms of action are largely unknown. In the past, IGSF1 (also known as InhBP/p120) was proposed as a pituitary receptor for inhibin B, and a regulator of follicle-stimulating hormone (FSH) expression[7,8]. However, physical interaction between inhibin B and IGSF1 could not be demonstrated by ligand-receptor binding[9].

Male and female *Igsf1* knockout mice were reported to have normal phenotype, gonadotropin levels and fertility, leaving a putative role of IGSF1 on the sex hormone axis uncertain[10]. After identification of the human phenotype of IGSF1 deficiency, detailed phenotyping confirmed that *Igsf1*$^{(-/-)}$ mice had reduced TSH pituitary content and serum TSH[4]. However, pituitary Tshb mRNA expression was reported normal. Therefore, the molecular function and implications of IGSF1 on gonadal and thyroid hormone axes remain to be elucidated. Knowing such mechanisms will be valuable to explain the large phenotypic variability of patients with IGSF1 defects and to define the physiological pathways disrupted in the disorder[4,11]. Notably, the typical testicular enlargement can be apparently absent in some IGSF1-deficient patients[12,13] while a variable presence of partial deficiency of growth hormone (GH) and prolactin was reported in a few others[4,11]. Finally, IGSF1 is present in different tissues, with predominant expression in pituitary and testis[6,7,14]. However, both tissues are cellularly heterogeneous, and cell type-specific expression of IGSF1 needs to be defined, especially in the pituitary, where contradicting results in rodents (rat and mouse) leave expression of Igsf1 in gonadotropes in the uncertain[4,7].

Here we present the detailed, longitudinal and long-term phenotype (from neonate to adult) of the original patient in whom the disorder was clinically described[3], harboring a complete deletion in *IGSF1*. We unveiled the cell-type specific expression of the IGSF1 protein in rat pituitary (thyrotropes and gonadotropes) and in human and mice testis (Leydig and Germ cells). We further show that IGSF1 has divergent transcriptional effects on two different pituitary gene promoters. IGSF1 potentiates transcription of the human thyrotropin-releasing hormone receptor (*TRHR*) promoter by repressing the TGFβ1-Smad pathway, a signal which is negatively modulating *TRHR* expression. However, IGSF1 negatively modulates the transcription of the human *FSHB* gene promoter through direct inhibition of the activin-Smad pathway. Clinical, immunohistochemical and molecular correlates of the study suggest that the two main features of the human IGSF1 deficiency may both originate from major pituitary abnormalities. This work unravels a crucial role of IGSF1 as an important regulator of TGFβ superfamily pathways in the pituitary.

## Results

**Clinical case.** The patient is a male of Spanish descent, born to unrelated parents. He was not detected by the TSH-based Neonatal Screening Program, but presented with severe clinical hypothyroidism 14 days after birth, including typical myxedematous face, protruding tongue and lethargy (Fig. 1A). He had low serum free T4 and inappropriately low-normal serum TSH (Table 1). Thyrotropin-releasing hormone (TRH) stimulation test confirmed central hypothyroidism with a poor TSH response and normal prolactin secretion (Fig. 1B). The mother of the patient had abnormally low TSH (0.02 mIU/L) and FT4 in the normal range (19.3 pmol/L). The father showed unremarkable endocrine phenotype (Table 2).

The patient was started on standard levo-thyroxine (L-T4) replacement at 15 days of life. With advancing age, unusually low L-T4 doses were needed to maintain euthyroidism (Table 1), as described in Central Hypothyroidism as compared to Primary Hypothyroidism[15].

The other pituitary axes were intact in the patient, including normal basal GH levels and adrenocorticotropic hormone (ACTH) dynamic test (Synacthen®) (Supplemental Fig. 1A and B). Brain MRI showed normal size and shape of the pituitary. Thyroid ultrasounds and scintigraphy at 6 years of age showed eutopic, mildly hypoplastic thyroid of reduced echogenicity and isotopic uptake (data not shown).

At 14 days of life, patient's sex hormone profile revealed an abnormal elevation of FSH compared to normal male newborns. FSH continued elevated during "mini-puberty", a physiological period when the gonadal axis is transiently active[16,17] (Table 1). At the end of mini-puberty (5 months of age) when the axis becomes inactive, patient's FSH returned to normal levels. Luteinizing hormone (LH) was not elevated and testosterone was normal (Table 1). From 3 years onwards, the patient developed a clearly progressive bilateral and symmetric macroorchidism under normal prepubertal values of FSH, LH, and testosterone (Table 1 and Supplemental Fig. 2). A gonadotropin-releasing hormone (GnRH) test was performed at the age of 6 years showing abnormally increased FSH and LH levels in ratios suggesting initiation of puberty. However, the expected increased in testosterone was not present, ruling out the start of a precocious puberty (Fig. 1C). Testosterone levels remained low until his puberty spontaneously started at a normal age of 12 years, but yet with enlarged testicular volume (12 ml), as measured by orchidometer (Table 1 and Supplemental Fig. 2).

At 14 years, the patient was extensively characterized by an additional TRH test and complete sex and thyroid hormone profiles. L-T4 withdrawal confirmed permanent central hypothyroidism with TSH in the normal ranges and low total and free T4 and T3 (Table 2). A mild improvement in the TSH peak was detectable in response to TRH through age (Supplemental Fig. 1C). Testosterone, estradiol, FSH and the common alpha-subunit (CGA) were normal and LH levels slightly decreased (Table 2). Remarkably, inhibin B and anti-müllerian hormone

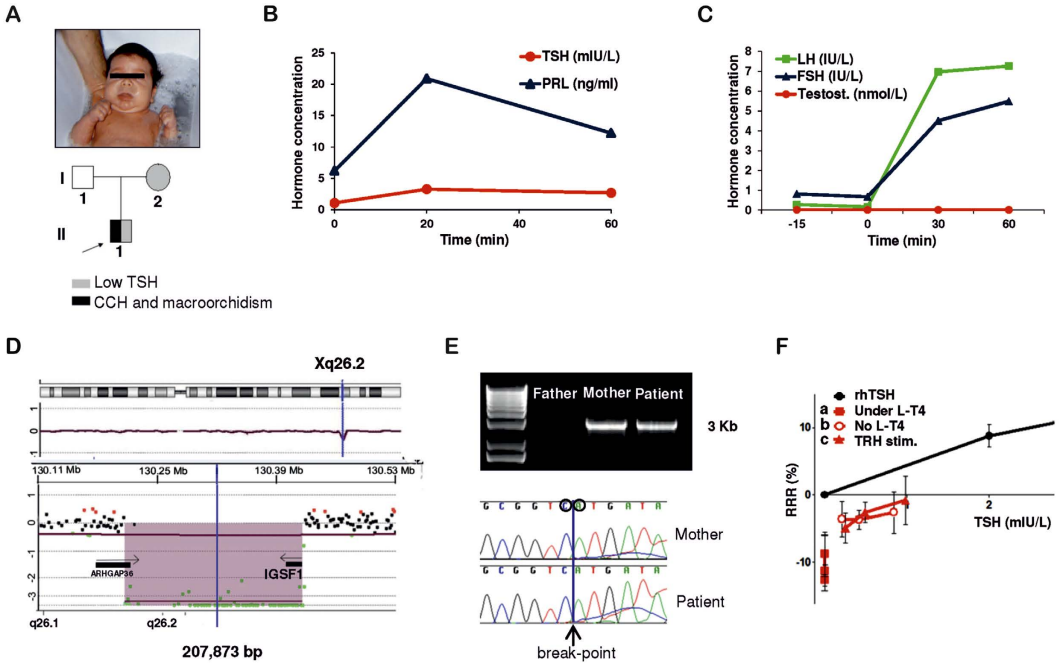


**Figure 1. Clinical features and genetic studies of the patient with IGSF1 deletion.** (**A**) Picture of the 14 day-old-patient with severe phenotype of hypothyroidism including facial myxedema and protruding tongue. The boy was born to a phenotypically normal father and a euthyroid mother with low TSH. (**B**) TRH stimulation test at 3 years of age. Low TSH response (peak = 3.2 mIU/L) and normal prolactin response consistent with pituitary defect[68]. (**C**) GnRH test at 6 years of age. Abnormal over-stimulation of FSH and LH and unresponsive testosterone without development of clinical signs of precocious puberty, excepting macroorchidism[69]. (**D**) CGH-Array showing the deletion of 207 Kb in Xq26 including the *IGSF1* gene. (**E**) PCR-amplified DNA fragments using primers on the flanking regions of the deletion in non-carrier father, heterozygous mother and hemizygous patient. A band of around 3 Kb is amplifiable when the deletion is present. Precise break-point of deletion as sequenced in PCR fragments from mother and patient. (**F**) TSH bioactivity of patient's serum at the age of 14 (red) in relation to standard rhTSH (black): (a) patient's serum under L-T4; (b) patient's serum after four weeks of levo-thyroxine withdrawal; (c) patient's serum after TRH stimulation test. Three dilutions for each condition are shown: 1:2, 1:4 and 1:8. CCH: central congenital hypothyroidism, min: minutes, TSH: thyrotropin, PRL: prolactin, FSH: follicle-stimulating hormone, Testost.: testosterone, GnRH: gonadotropin-releasing hormone, rhTSH: recombinant human TSH, L-T4: levothyroxine treatment, TRH stim.: TRH stimulus, RRR: Relative Response Ratio.

| Age | *14 d | 1 mo | 5 mo | #3y | #6y | 8 y | 9 y | 10 y | 11 y | 12 y | 12.5y | 14 y |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| TSH *mIU/L* | 1.4 | *0.11* | *0.09* | 1.07 | 1.52 | *<0.01* | *0.08* | *<0.01* | *<0.01* | *<0.01* | *<0.01* | *<0.01* |
| FT4 *pmol/L* | *7.2* | 26 | 23.5 | *9.9* | *7.7* | 16.1 | 11.6 | 14.8 | 11.2 | *8.4* | 13.5 | 11.7 |
| L-T4 dose *μg/Kg/day* | 0 | 10 | 5 | 0 | 0 | 1.9 | 2 | 1.65 | 1.4 | 1.35 | 1.9 | 1.4 |
| FSH *IU/L* | **6.3** | **4.5** | 1.2 | 0.43 | 0.66 | 0.74 | 0.88 | 1.10 | 0.82 | 1.11 | 1.76 | 2.56 |
| LH *IU/L* | *0.15* | na | 0.54 | <0.1 | 0.2 | 0.10 | 0.14 | 0.18 | 0.10 | 0.27 | 0.85 | 0.41 |
| Testosterone *nmol/L* | 1 | na | 1 | na | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 | 0.6 | 13.50 | 11.80 |
| †Testicular vol. *ml* (*p98 upon age*) | Na | 2 (*2.8*) | 2 (*2.8*) | **3** (*2.8*) | **4** (*3.5*) | **5** (*3.8*) | **5** (*4*) | **6** (*5*) | **8** (*8*) | **12** (*15*) | **20** (*24*) | **40** (*28*) |

**Table 1. Long-term follow up of thyroid and gonadal hormone profiles of the patient with IGSF1 deletion.** Hormone parameters are presented in chronological order along patient's lifespan. In italics and bold, hormone values below and above normal ranges for age, respectively. *Age at clinical diagnosis of the patient. #Age after 1 month levo-thyroxine withdrawal to perform two separate TRH tests. †Testicular volume was measured by orchidometer until size exceeded 25 ml, when a caliper and the Lamber's formula were used[18]. TSH: thyrotropin, FT4: free thyroxine, L-T4: levothyroxine treatment, FSH: follicle-stimulating hormone, LH: luteinizing hormone, vol.: volume. d: days; mo: months; y: years; na: not available. Normal ranges for TSH and FT4 are from the Laboratory of the Institution where determinations were performed (TSH: 0.4–4.3 mIU/L, FT4: 11–25 pmol/L), age-related normal ranges for gonadal hormones are from refs 73–75, and for testicular volume are from ref. 52.

(AMH) were strongly elevated, indicating an increased number of Sertoli cells in the testis. At this stage, testicular volume was yet 40 ml (normal: 15–25 ml, Supplemental Fig. 2), and final adult volume reached 50 ml (60 × 40 mm, Supplemental Fig. 2), as calculated using a caliper and the Lamber's formula[18]; testicular ultrasounds showed

| | Patient | | | | | Normal ranges | | |
|---|---|---|---|---|---|---|---|---|
| | A | B | C | Mother[1] | Father | Male | Male | Female |
| Age | 14 y | 14 y | 14 y | 50 y | 49 y | T-IV | Adult | Menopause |
| TSH *mIU/L* | *0.002* | 1.7 | 2.0 | *0.002* | 2.17 | *0.4–4.3* | | |
| FT4 *pmol/L* | 17.2 | *8.3* | *9.1* | **26.6** | 17.1 | *11–25* | | |
| T4 *nmol/L* | 67 | *41.4* | *40.2* | 109.0 | 95.1 | *58–128* | | |
| T3 *nmol/L* | 1.57 | 1.37 | 1.40 | 1.82 | 1.58 | *1.4–2.5* | | |
| L-T4 dose *μg/Kg/day* | 1.4 | 0 | 0 | 1 | 0 | | | |
| FSH *IU/L* | 2.9 | 4.0 | 4.1 | 123.0 | 3.8 | *0.3–8* | *1.5–14* | *35–150* |
| LH *IU/L* | 0.6 | 1.0 | 1.1 | 32.8 | 3.4 | *0.5–5* | *1.5–8* | *15–90* |
| SHBG *nmol/L* | 20.3 | 11.7 | 11.7 | 78.8 | 31.7 | *13–88* | *10–70* | *20–120* |
| Testosterone *nmol/L* | 11.3 | 15.3 | 11.0 | *0.1* | 11.7 | *9–19* | *10–30* | *0.5–3* |
| E2 *pmol/L* | 97 | 112 | 85 | 27 | 104 | *17–178* | *50–200* | *<50* |
| Inhibin B *ng/L* | **505** | **423** | **500** | 20 | 236 | *107–310* | *150–400* | *10–200* |
| AMH *μg/L* | **44.4** | **48.0** | **40.7** | 0.1 | **15.3** | *2–15.5** | *1.8–13.7** | *<0.1* |

**Table 2. Detailed thyroid and gonadal hormones profiles of the IGSF1-deficient patient at 14 years of age and his parents.** Three hormone profiles of the patient correspond to three subsequent time points: A, Under L-T4 treatment, B, Four weeks after levo-thyroxine withdrawal for the performance of a TRH test, and C, 3 hours after TRH stimulation. In italic and bold, hormone values below and above normal ranges, respectively. [1]The mother started levo-thyroxine treatment for central hypothyroidism 1 year after diagnosis of her child with hypothyroidism. Therefore, her hormone determinations were performed under L-T4 treatment. TSH: thyrotropin, FT4: free thyroxine, T4: total thyroxine, T3: total triiodothyronine, L-T4: levo-thyroxine treatment, FSH: follicle-stimulating hormone, LH: luteinizing hormone, SHBG: sex hormone-binding globulin, E2: estradiol, AMH: anti-müllerian hormone, y: years, T-IV: Tanner IV. Normal ranges for thyroid hormone axis and for adult male and postmenopausal female gonadal hormones are from the Laboratory of the Institution where determinations were performed. Normal ranges for gonadal hormones of a male in the Tanner IV stage are from refs 73–76. *Normal range for male anti-müllerian hormone is from ref. 77.

normal echogenicity, ruling out tumor-related growth. Sperm production was preserved with normal amount (100 million/ml) and mobility of spermatozoids at 15 years of age (data not shown).

**Complete deletion of IGSF1 gene.** Direct sequencing of the coding exons and exon/intron boundaries of three candidate genes for central hypothyroidism (*TSHB*, *CGA* and *TRHR*) revealed no genetic abnormalities.

Comparative Genomic Hybridization (CGH)-Array showed a hemizygous deletion on chromosome X in the patient. The deleted region included the entire *IGSF1* gene sequence (Fig. 1D) and the break-point was studied in the patient and parents using long PCR and Sanger sequencing, revealing a 207.873 Kb deletion in Chr. Xq26.2 (chrX: 130,217,339–130,425,212 GRCh37/hg19), present also in the mother in heterozygote state (Fig. 1E). The whole IGSF1 gene is deleted, including all putative 3′ regulatory regions and the 5′ promoter.

The ARHGAP36 gene is deleted in its 3′-coding and putative 3′regulatory regions. The promoter is conserved. Very little is known about the function and expression pattern of this gene[19]. Its lack of expression has not been linked to any specific animal or human phenotype. Based on the information available, this gene does not seem to have an effect on the phenotype we described, which exactly coincides with that of the typical IGSF1 defect in humans, without the presence of any other additional sub-phenotypes.

**TSH bioactivity is low in IGSF1 deficiency.** TSH is one of the pituitary hormones that are glycosylated. This glycosylation is essential for TSH activity. The hypothalamic hormone TRH acts in the pituitary to regulate not only TSH expression but also its correct maturation and bioactivity (glycosylation)[20,21].

Patient's TSH bioactivity was studied at the age of 14 years from three different serum samples taken: (a) under L-T4 treatment, (b) after four weeks of levo-thyroxine withdrawal prior to a TRH test and (c) 3 hours after TRH stimulation (Fig. 1F). TSH bioactivity was undetectable under L-T4 treatment (a), corresponding to very low immunoreactive TSH levels (Table 2). Four weeks after L-T4 withdrawal (b), serum TSH bioactivity was shown to be significantly decreased despite normal serum TSH quantitative values in the immunoassay (Table 2). TSH bioactivity and quantitative values did not improve after stimulation with TRH (c) (Fig. 1F). This shows that patient's TSH is not only inappropriately normal, but also that its bioactivity is markedly decreased, suggesting abnormal glycosylation pattern of the TSH[20]. The serum TSH of the patient's mother (carrier of the IGSF1 deletion) also had decreased bioactivity, whereas the father's TSH bioactivity was normal (Supplemental Fig. 3A).

**IGSF1 is located in rat pituitary thyrotropes and gonadotropes.** To evaluate the presence of IGSF1 in the anterior pituitary, western blot in human and rat pituitary extracts and double immunofluorescence with confocal co-localization of rat Igsf1 and each of the pituitary hormones were performed. IGSF1 is abundantly expressed in human and rat adenopituitary by western blot using a commercial antibody, and the major band was of the expected size (around 148 kDa) (Supplemental Fig. 4A). In double immunofluorescence of rat pituitary sections, Igsf1 was co-expressed in all TSH beta positive (+ve) thyrotropes, both in their top-middle and lobe locations of the adenopituitary (Fig. 2A and Supplemental Fig. 4B). Quantitative analysis showed that 90.3%

of Igsf1 +ve cells in the pituitary top-middle portion co-expressed TSH in comparison with only 63.9% of the of Igsf1 +ve cells in the lobes. This is consistent with the top-middle pituitary being an area enriched in thyrotropes. Similarly, all gonadotropes (FSH beta +ve or LH beta +ve) expressed Igsf1 in their pituitary lobe location (Fig. 2A and Supplemental Fig. 4B).

Consistent with these results, Igsf1 also co-localized with all cells expressing alpha Glycoprotein Subunit (aGSU), the common subunit for thyrotropic and gonadotropic hormones (Fig. 2B and Supplemental Fig. 4B–D). In contrast, Igsf1 was not present in somatotropes (GH +ve), lactotropes (PRL +ve) or corticotropes (ACTH +ve) (Fig. 2B and Supplemental Fig. 4B). Structurally, although Igsf1 was not an exclusive plasma membrane protein, the Igsf1 staining showed membrane reinforcement and colocalized with E-cadherin indicating a function related to the plasma membrane (Supplemental Fig. 4E). These results are in contrast with previous results by Joustra *et al.* using a different antibody that localized Igsf1 in Pit-1 expressing cells within the rat pituitary, while SF-1/Lh expressing cells did not co-localize with Igsf1[22].

To confirm the cell-specific pattern of expression of Igsf1 in the pituitary we measured Igsf1 mRNA expression in each type of endocrine cell. We purified enriched populations of somatotropes, gonadotropes and thyrotropes from cell dispersions of young adult male rat pituitary, using antibodies anti-Gh, -Fshb, and -Tshb respectively and immune-magnetic purification (Fig. 2C). qRT-PCR was performed in those purified populations to quantify mRNA expression for *Gh*, *Fshb*, *Tshb and Igsf1* together with the control gene 18 s. As expected, in each of the purified populations there was an enrichment of the mRNA for the corresponding hormone: Gh in somatotropes, Fshb in gonadotropes and Tshb in thyrotropes, although this one was less enriched since the number of thyrotrophs in a normal pituitary is less than 10% of the endocrine population[23,24]. Igsf1 mRNA was enriched in the gonadotrope and thyrotrope population (Fig. 2C). However, we were unable to detect Igsf1 mRNA in the somatotroph population. This result agrees with the above confocal studies and, together with the western blot, validates our immune co-localization pattern of Igsf1.

***IGSF1* is mainly present in germ cells and Leydig cells of human and mice adult testis.** To investigate the presence of IGSF1 in testis, we used immunohistochemistry and double immunofluorescence with confocal co-localization of IGSF1 and three cell-type specific testicular markers (Calretinin, Inhibin and Melan A). Three different samples of human testicular tissue (from donor men of 42, 52 and 73 years of age) were stained for IGSF1 and compared with the typical staining pattern of Leydig cells (Calretinin), Sertoli cells (Inhibin) or both (Melan A) (Fig. 3A). IGSF1 signal was present within and outside the seminiferous tubules. In the interstitium, IGSF1 staining was similar to those typical of Calretinin and Melan A (Fig. 3A*d*,*f*), indicating the presence of IGSF1 in Leydig cells. Within the tubules, IGSF1 stained cells from the basement to the lumen, with reinforced staining of basal and peri-luminal layers (Fig. 3A*a*–*c*). This pattern differs from that typical of Inhibin - strong staining in basal "spikes", inter-spermatogonia, and no peri-luminal staining - and that of Melan A in the tubules (Fig. 3A*e*,*f*), suggesting that IGSF1 could be absent or have very low expression in human adult Sertoli cells while being present in the germ epithelium.

To further investigate IGSF1 in testicular cell populations, double immunofluorescence for IGSF1 and Calretinin or Inhibin, respectively, was performed. IGSF1 was again detected from basement to lumen within the seminiferous tubules, more intensely at basal and peri-luminal areas (Fig. 3B*a*–*d*). IGSF1 co-localized with Calretinin in Leydig cells, although the staining was less intense than in the tubules (Fig. 3B*a*,*b*,*f*–*i*). Within the seminiferous tubule, double-fluorescence of Inhibin and IGSF1 showed distinct locations and no co-localization, indicating that Sertoli cells are clearly different from the cell population expressing IGSF1 (Fig. 3B*c*,*d*,*j*–*m*) and may not express IGSF1. On the other hand, faint and scarce yellow (co-localization) spot signals could be rarely detected (Fig. 3B*c*,*d*,*l*,*m*). These few yellow spots may correspond either to crossing points between the two layers of cells (germ cells and Sertoli cells) or to Sertoli cells expressing some IGSF1 only in few restricted contact areas with the germ cells.

Therefore, expression of IGSF1 in human adult testis is concentrated mainly the germ cell epithelium, being especially intense in the cytoplasm of spermatogonia. It is also present in Leydig cells and has very low or no expression in adult human Sertoli cells.

Parallel experiments in adult mice testis revealed similar patterns of Igsf1 staining and cell-type specific expression compared to those in human testis (Supplemental Fig. 5), being mainly present in germ cells and Leydig cells. Remarkably, mouse germ epithelium presents a distinct staining of germ granules not found in humans.

**IGSF1 activates the TRHR promoter and represses the FSHB promoter.** Our patient presented with reduced TSH secretion combined with reduced serum TSH biopotency. Both processes involve TSH beta (*TSHB*) gene expression and TSH glycosylation respectively, and both are known to be dependent on TRHR activation[20,21]. On the other hand, the patient presented with excessive FSH secretion as a neonate which, interestingly, resulted in macroorchidism, as happens in FSH-secreting pituitary adenomas[25–27]. FSH secretion is critically regulated by testicular inhibin B through inhibition of the stimulatory activin-Smad signaling pathway, and IGSF1 was proposed as a putative inhibin B receptor[7]. Based on the evidence that *FSHB* is activated by the activin-Smad pathway[28–30], the presence of putative Smad-responsive elements in the *FSHB* and *TRHR* promoters was investigated *in silico*. In the human *FSHB* promoter a putative Smad-responsive element (CTGTCTATCTA) at −133 to −123 bp and a putative Smad3-binding element (AGGCAGCCG) at +17 to +25 from transcription start site were identified. In the human *TRHR* promoter a putative Smad3-binding element (AGACAGATA) was identified between positions −1173 and −1165 bp from transcription start site. Although an alteration of the GnRH Receptor could also be involved, it has been demonstrated in rat and mouse models that *Gnrhr* expression in gonadotropes is fully independent of Activin signaling[31].

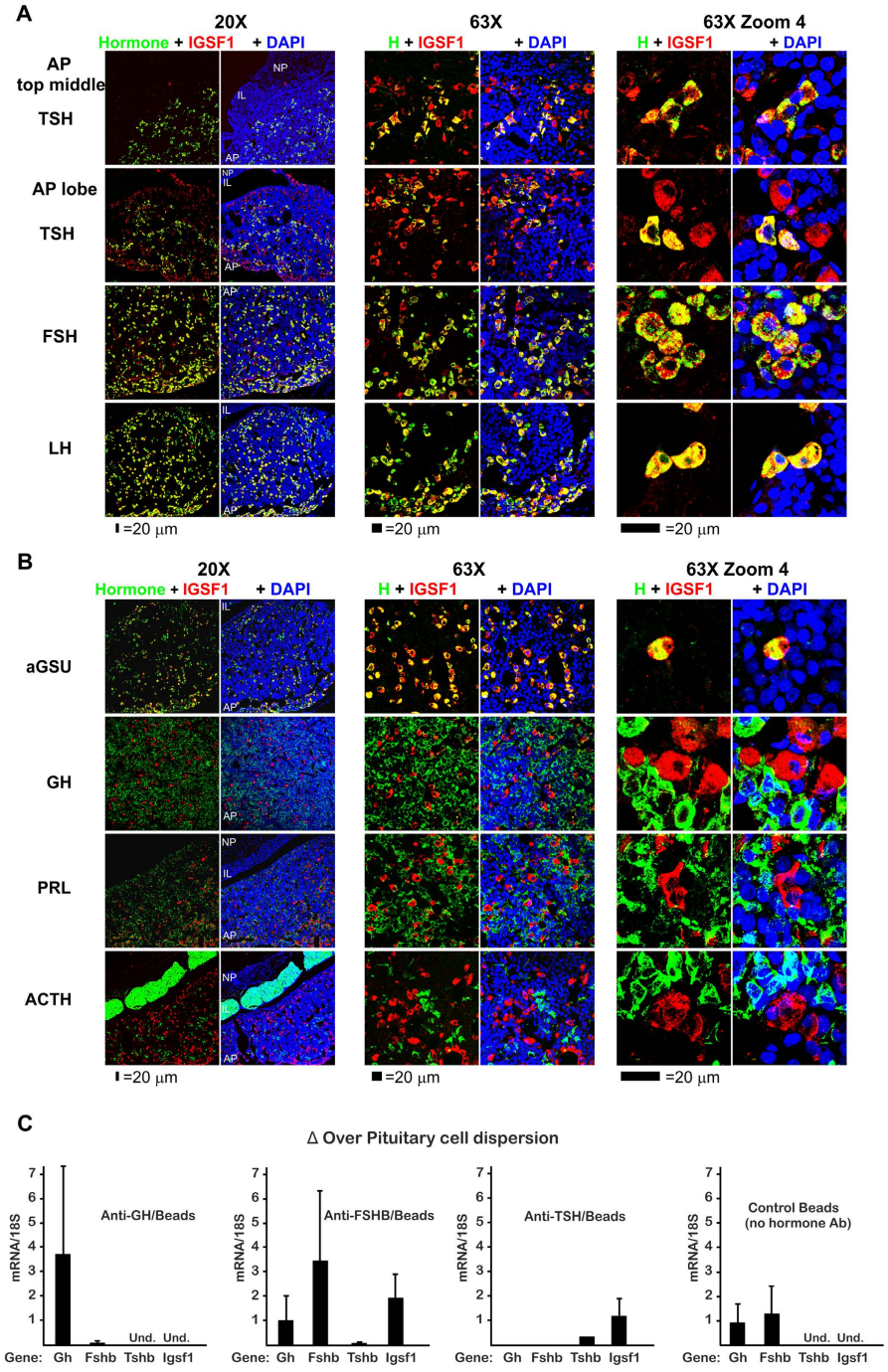


**Figure 2. IGSF1 cellular expression in young adult male rat pituitary gland is expressed in thyrotrope and gonadotrope endocrine cells.** Coronal pituitary sections were stained and topographical serially studied using confocal microscopy from the middle towards the lobes at different magnifications (20X, 63X and 63XZoom4). A white laser was used to prevent differences in intensity by use of different wavelength lasers. IGSF1 is shown in red pseudocolor while every hormone is shown in green. Nuclei were stained with DAPI and shown in blue. (**A**) IGSF1 is located exclusively in the AP but not in the IL or the NP and co-localizes with the three hormones: TSH beta, FSH beta and LH beta. The number of double positive IGSF1/TSH was higher in the middle of the section (thyrotrope region) than toward the lobes (gonadotrope region). (**B**) All IGSF1 cells co-localize with aGSU, the common alpha subunit for TSH, FSH and LH. No somatotrope (GH), lactotrope (PRL) or corticotrope (ACTH) was found to be expressing IGSF1. Quantifications are shown in Supplemental Fig. 2B. (**C**) Immune-magnetic purification of somatotropes (anti-GH/Beads), gonadotropes (anti-FSHB/Beads) and thyrotropes (anti-TSHB/Beads) from a single cell dispersion of rat pituitary followed by qRT-PCR. Results are expressed as enrichment (gene mRNA/18S) over the initial cell dispersion. As a control, magnetic beads in the absence of hormone antibody were used. As expected, Gh mRNA was enriched in anti-GH, Fshb mRNA in anti-FSHB and Tshb mRNA in anti-TSHB purified cells. Igsf1 mRNA was detected exclusively in the anti-FSHB and anti-TSHB purified cells. Results are the mean of four independent experiments. AP = adenopituitary. IL = intermediate lobe. NP = neuropituitary.

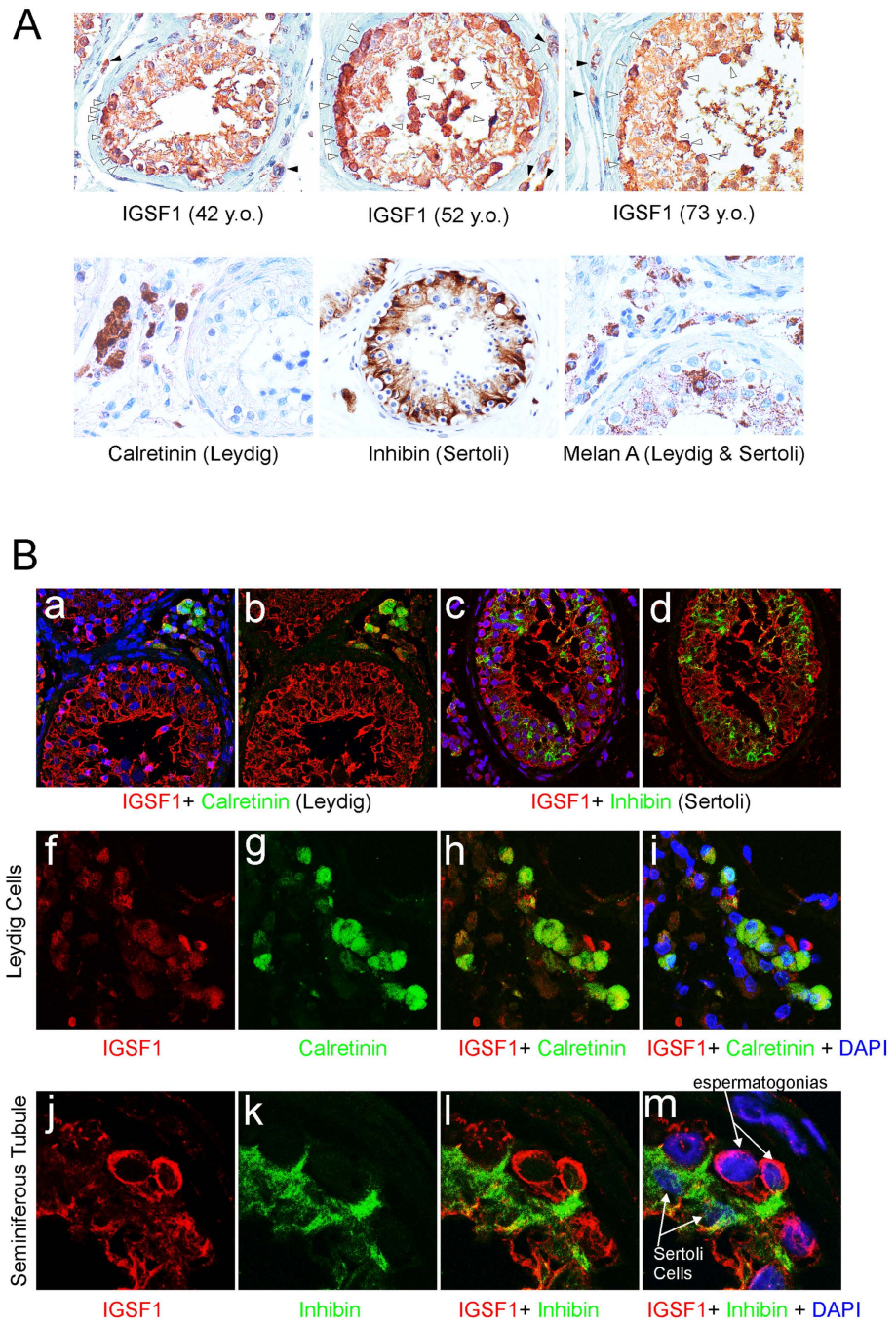


**Figure 3. IGSF1 stain germ cells and Leydig cells in human adult testes.** (**A**) Immunohistochemistry of IGSF1 in a tissue array of three independent samples of human testes (***a–c***, 42, 52, and 73 years old). Staining is seen inside the whole wall of tubules with stronger staining at the basal layer and also at the lumen (white arrowheads). In the interstitium, some peripheral cells are also stained (black arrowheads). The array was also stained for markers of Leydig cells (Calretinin), Sertoli cells (Inhibin) or both (Melan A) (***d,e,f***, respectively). IGSF1 positive peripheral cells could correspond with Calretinin or Melan A staining outside the tubules. However, inside the tubule, Inhibin or Melan A (both present in Sertoli cells) and IGSF1 do not seem to stain the same populations. (**B**) To further study IGSF1 cell populations double immunofluorescence for IGSF1 + Calretinin (***a,b***) and IGSF1 + Inhibin (***c,d***) was studied using confocal microscopy. Calretinin (Leydig cells, green) co-localizes with IGSF1 (red) at the periphery of the tubules, although they stain different cell areas. This is further seen when zooming in (***f–i***). Opposite, inside the tubule Inhibin does not seem to co-localize with IGSF1 (***c,d***). IGSF1 (red) is strong at the periphery and lumen while Inhibin appears in specific areas of the tubule wall as corresponding to its tree-like form. In ***j–m*** a zoomed area is observed with two marked spermatogonia stained for IGSF1 (red) and two Sertoli cells stained for Inhibin (green). Sertoli arms surround the IGSF1 positive spermatogonia and are negative for IGSF1. The few yellow spots that could be observed in ***c,d*** and ***l,m*** may correspond either to crossing points between the two layer of cells (germ cells and Sertoli) or to Sertoli cells expressing IGSF1 only in few and restricted contact areas.

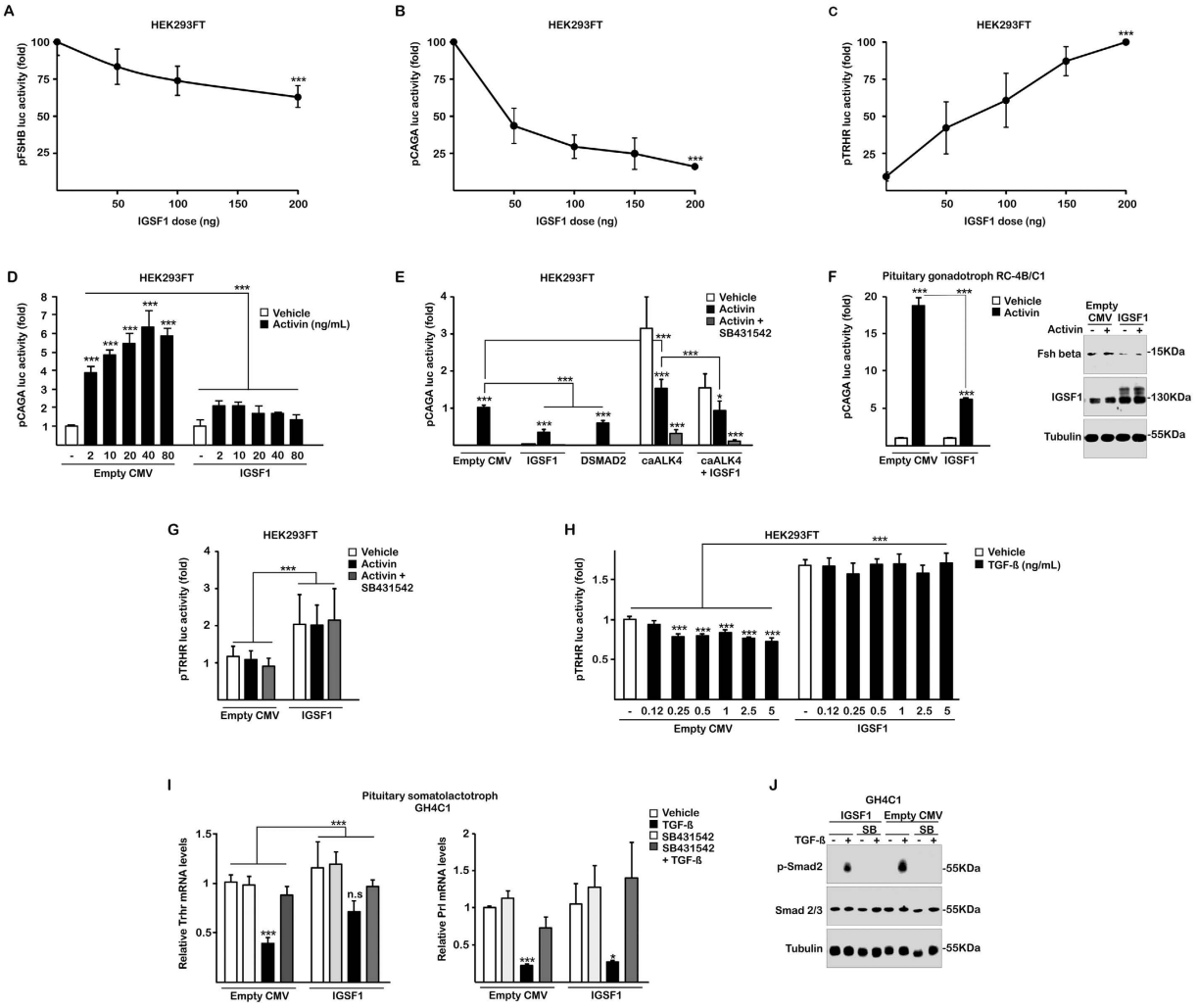


**Figure 4. Molecular mechanisms and pathways involved in IGSF1 deficiency.** In HEK293FT, IGSF1 represses the activity of both a human *FSHB* −875/+120 minimal promoter (*pFSHB*) (**A**) and its potent surrogate *CAGA-luc* promoter (*pCAGA*)(**B**), in a dose-response manner. (**C**) IGSF1 stimulates basal human *TRHR* promoter (*pTRHR*) activity in a dose-dependent manner. (**D**) A dose-response curve of Activin (2–80 ng/ml) demonstrates the potency of IGSF1 blunting the response to Activin. (**E**) IGSF1 is more potent than a dominant negative SMAD2 mutant (DSMAD2) and is able to repress the constitutive activity of an activating mutant of the Activin receptor (caALK4) in the absence of Activin. SB431542, antagonist of the receptor, was added in all conditions to show the functionality of the pCAGA response, blunted when this inhibitor was present. (**F**) In pituitary gonadotropes, RC-4B/C1, pCAGA response to activin was huge -circa 18 times over control- and reduced to 5 times in the presence of IGSF1 (left). A western blot shows induction of Fshb expression with Activin that was blunted in the presence of IGSF1 (right). (**G**) Activin does not induce the human *TRHR* promoter nor alters the basal stimulation in the presence of IGSF1. (**H**) TGFβ represses the luciferase activity of the *TRHR* promoter in a dose-response manner. Repression is reversed by IGSF1. (**I**) Endogenous expression of Trhr in pituitary GH4C1 cells by qRT-PCR (left). Trhr mRNA is repressed by TGFβ (black bars) and this is reversed by the TGFβ-inhibitor SB431542 (dark grey bars). IGSF1 blocks such repression as efficiently as the inhibitor. Prolactin (Prl) mRNA, another gene repressed by TGFβ, was not affected by the presence of IGSF1 and was equally repressed by TGFβ in its presence. SB431542 blocked TGFβ action in both genes. (**J**) C-terminal phosphorylation of SMAD2 (p-Smad2), mediated by TGFβRII/RI complex upon TGFβ addition, was markedly reduced by IGSF1 in pituitary GH4C1. Extracts were taken after one hour with TGFβ or Vehicle. SB431542, the TGFβRI inhibitor, blocks p-SMAD2 independently of the absence/presence of IGSF1. n.s.: not significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$.

We tested the hypothesis that IGSF1 acts on pituitary regulation of *FSHB* and *TRHR* gene expression. Transfection of increasing concentrations of IGSF1 in the presence of a fixed dose of activin A significantly decreased in a dose-dependent manner the transcriptional activity of a −875 to +120 bp human *FSHB* minimal promoter (minimal *FSHB* promoter) cloned in house (Fig. 4A). We also tested an artificial activin-sensitive promoter (CAGA) containing twelve Smad3-binding motives[32], previously used in Lbt2 gonadotropic cells yielding a potent transcriptional activity in response to activin, 10-fold higher than that of the *FSHB* promoter[33]. IGSF1

strongly and dose-dependently reduced the activity of the CAGA promoter in presence of a fixed dose of activin A (Fig. 4B). For further experiments CAGA was used as a potent surrogate of the natural human *FSHB* promoter. On the other hand, we used a luciferase-reporter containing the human *TRHR* promoter[34]. Co-transfection of increasing concentrations of IGSF1 resulted in increased basal activity of the *TRHR* promoter, in a dose-dependent manner (Fig. 4C). Therefore, IGSF1 exerts opposite transcriptional effects over two critical gene promoters in pituitary physiology, namely activation of *TRHR* in thyrotropes and repression of *FSHB* in gonadotropes.

**The activin pathway mediates the effects of IGSF1 on the CAGA promoter, but not those on the TRHR promoter.** To further investigate the effects of IGSF1 on Activin-Smad signaling, we performed a dose-response curve of Activin on the CAGA transcriptional activity in the absence/presence of fixed amount of IGSF1 (Fig. 4D). IGSF1 exerted a potent inhibitory effect, reducing responses to Activin more than six-fold (Fig. 4D). The activin effect was abolished by SB431542, an inhibitor of activin type I receptors, independently of IGSF1 (Fig. 4E). Furthermore, IGSF1 was more potent than DSmad2, a truncated protein with dominant negative effect over the wild type Smad2, which reduced CAGA activity by 40% in comparison with the 70% reduction of IGSF1 (Fig. 4E)[35]. ALK4, the type I activin receptor, is expressed in gonadotropes[36]. Transfection of a constitutively active (ca) mutant (caALK4) increased three-fold the basal transcriptional activity of CAGA in the absence of activin A (Fig. 4D). Expression of IGSF1 was also able to reduce such unstimulated caALK4-dependent transcriptional activity by 50% (Fig. 4D). These results indicate that IGSF1 is a strong repressor of the activin pathway on the CAGA promoter through a mechanism involving the ALK4 receptor. Although IGSF1 repressed the basal activity (Fig. 4A), we were unable to detect induction of this minimal human FSHB promoter by Activin in these cells (data not shown). It is known that Activin induction of the human *FSHB* gene in gonadotropes requires far upstream 5′ and downstream 3′ sequences (on exon 2 and exon 3) that were absent in our construct[31,37].

As a model of pituitary gonadotrope we use the rat pituitary cell line RC-4B/C1, described as presenting a majority of cells staining for FSH and LH protein expression[38]. In those gonadotrope cells, Activin induced a strong increase in CAGA activity of near 20-fold that was reduced to 5-fold in the presence of IGSF1 (Fig. 4F). As expected[31,37], we were again unable to detect induction of the minimal human *FSHB* promoter by Activin in these RC-4B/C1 gonadotrope cells, although IGSF1 repressed the basal activity (data not shown). On the other hand, we studied the intracellular protein expression of Fsh beta using western blot (Fig. 4F). Fshb was induced by Activin, and repressed when IGSF1 was transfected, confirming in a direct way the repression of Fshb protein expression by IGSF1 in gonadotropes (Fig. 4F).

**TGFβ1 pathway mediates the effects of IGSF1 over the TRHR promoter.** A parallel set of experiments was performed using the human *TRHR* promoter as target of IGSF1 effects. However, neither Activin A nor SB431542 influenced the stimulatory effect of IGSF1 on the basal activity of the *TRHR* promoter (Fig. 4G). Similarly, neither the inhibitory DSmad2 nor the constitutively active ALK4 receptor (caALK4) altered IGSF1 stimulation of *TRHR* promoter (Supplemental Fig. 6). These results indicate that IGSF1 action on the human *TRHR* is independent of activin A and must be mediated by an alternative signaling cascade.

TGFβ pathways are active in multiple processes of pituitary development, differentiation and function. TGFβ 1 is known to be expressed in lactotropes, gonadotropes and thyrotropes[39]. Since prolactin secretion is negatively modulated by TGFβ1 and positively regulated by TRHR[40], we tested the hypothesis that TGFβ1 could down-regulate *TRHR* expression and, in turn, IGSF1 could counteract this effect. HEK293FT cells were transfected with the *TRHR* promoter construct and treated with different doses of TGFβ1 in the absence or presence of IGSF1 (Fig. 4H). TGFβ1, from 0.25 to 5 ng/ml, significantly reduced *TRHR* transcriptional activity but this effect was not only abolished but completely reversed in the presence of IGSF1 (Fig. 4H).

To confirm such IGSF1 effect on the endogenous *TRHR* gene, we transfected IGSF1 in rat pituitary GH4C1 cells, a somato-lactotrope cell line endogenously expressing Trhr, and measured Trhr mRNA expression by qRT-PCR (Fig. 4I). Indeed, TGFβ1 could strongly repress *Trhr* expression, an effect blocked by the inhibitor SB431542. IGSF1 was able to block TGFβ1 repression, preventing the TGFβ1-induced down-regulation of endogenous *Trhr* expression. Interestingly, TGFβ1 was also able to repress *Prl* mRNA expression, but IGSF1 did not alter such repression (Fig. 4I). These results indicate that IGSF1 action on *TRHR* is mediated by a specific alteration on the TGFβ1-Trhr signaling pathway.

TGFβ binds to TGFβRII, the complex recruits and activates TGFβRI, which initially phosphorylates SMAD2 at Serine residues in the C-terminal tail, leading to modulation of other intracellular pathways[41]. Since IGSF1 is a plasma membrane protein and co-localizes with Cadherins (Supplemental Fig. 4), we explored the immediate signaling induced by TGFβ by measuring the phosphorylation of SMAD2 C-terminal tail (p-Smad2) in pituitary GH4C1 cells. We treated with TGFβ1 for one hour in the absence or presence of IGSF1. As expected, TGFβ1 caused a potent p-Smad2 induction that was blocked by the TGFβRI inhibitor SB431542 (Fig. 4J). IGSF1 markedly reduced p-Smad2 signal by 40% with respect to total SMAD3, or by 70% with respect to total loading of protein (Tubulin) (Fig. 4J). It is well known that Smads are weak transcription factors requiring a particular context of other transcription factors to repress/activate a particular gene within a cell[42,43] and this also happens in human endocrine cells[41]. We concluded that IGSF1 negatively regulates the TGFβ pathway, interfering with Smad2 activation which, in the thyrotrope intracellular context, leads to abrogation of the TGFβ effects on the *TRHR* gene.

## Discussion

IGSF1 deficiency is a recently identified human disorder of thyroid and gonadal hormone axes whose pathogenic mechanisms at the molecular level are not established.

Based on the long-term hormone follow-up of the patient in whom the disorder was originally described[3], we here unveil two independent transcriptional effects of IGSF1 in pituitary physiology, stimulation of TRHR

and repression of FSH synthesis, whose failure is mechanistically consistent with the hallmark features of human IGSF1 deficiency, central hypothyroidism and macroorchidism.

**Disruption of the thyroid hormone axis in IGSF1 deficiency.** Central Congenital Hypothyroidism (CCH) is usually regarded as a mild form of hypothyroidism. However, recent surveys show that it can be clinically severe[44]. Despite this, CCH is not routinely screened for in most countries, since detection of TSH elevations is the commonly used tool to diagnose (only) thyroidal hypothyroidism in babies. Our patient showed biochemically and clinically severe hypothyroidism at birth. Despite the early diagnosis (based on clinical manifestations) and treatment of his hypothyroidism, fine motor coordination deficits are present in the patient as an adult. Attention deficiency, although mild, has been previously described in affected male patients treated from birth[45,46]. This suggests prenatal, non-recoverable damage of brain development by *in utero* hypothyroidism in severe cases of IGSF1 deficiency. If undetected, neonatal hypothyroidism in the degree seen in our patient would likely have resulted in psychomotor retardation[44].

At diagnosis, free T4 was low but TSH was in the normal range, suggesting impaired TSH secretion and/or reduced TSH bioactivity. The latter was confirmed through a highly sensitive *in vitro* assay showing markedly decreased serum TSH biopotency against the TSH receptor. Together with the poor TSH response at the TRH test, this indicates that hypothyroidism in IGSF1 deficiency is a combination of quantitative and qualitative (glycosylation) defects of TSH secretion[20,21].

In fact, biological potency of TSH is determined by the glycosylation pattern generated on the molecule by pituitary enzymes (glycosyltransferases) whose activity is in turn controlled by TRH–TRHR signaling in thyrotropes[21]. TSH biopotency in our patient was not only reduced but also insensitive to TRH stimulation, which is consistent with our experimental findings that the ultimate molecular mechanism for hypothyroidism in this disorder is the decrease of *TRHR* expression.

We showed that the human *TRHR* gene promoter is transcriptionally repressed by TGFβ1, an active signaling pathway previously studied in primary cultures of pituitary cells[40]. In turn, this effect is negatively modulated by IGSF1 in a specific way since other genes regulated by TGFβ1 like *Prl* were not affected. IGSF1 modulates the immediate SMAD2 phosphorylation and activation at the plasma membrane, indicating that its blocking action is mediated, at least partially, through the SMAD pathway. Since SMADs are so heavily dependent on the intracellular context[41–43], a partial downregulation of SMAD2 activation could lead to abrogation of the TGFβ1 effects on the *Trhr* gene, while it is not enough to alter *Prl* repression. Another non-SMAD pathway that could be affected by IGSF1 is the Pit-1 regulation of the *TRHR* promoter[47]. Pit-1 is a major factor in thyrotropes, and there are two active biding sites in the proximal promoter of the *TRHR* gene. Future work is required to clarify whether Pit-1 regulation of TRHR is dependent on or enhanced by IGSF1.

IGSF1 is capable of increasing *TRHR* expression, and its deletion in our patient is consistent with a decrease in TRHR molecules at the membrane of thyrotropes, a known model for central hypothyroidism[1] (Fig. 5A). These results fully agree with the low Trhr mRNA levels observed in the pituitary of *Igsf1* deficient mice[4]. We identified here a mechanism whereby IGSF1 deficiency down-regulates endogenous TRHR, a pivotal molecule at the crossroad of TSH secretion signaling. Notwithstanding, Igsf1 mRNA was recently detected in the rat hypothalamus, mainly in glial cells (Gfap positive), but also in a small subset of TRH neurons[22]. Therefore, we cannot exclude other roles for IGSF1 at the hypothalamus that could also contribute to the phenotype of our patient. The intriguing possibility exists that hypothalamic Igsf1 contributes to the positive expression and pulsatility of Trh secretion and, thus, Igsf1 genetic defects could, in addition to reducing TRHR functionality in thyrotropes, decrease the hypothalamic trophic action on pituitary thyrotropes.

**Disruption of the gonadal hormone axis in IGSF1 deficiency.** We present here morphological, clinical and molecular evidence strongly indicating that a pituitary disorder may be an important pathogenic mechanism leading to macroorchidism in IGSF1 deficiency.

We show that IGSF1 is strongly expressed in gonadotropes of the young adult male rat enabling the "pituitary pathogenic hypothesis" for macroorchidism. Using an IGSF1 antibody against aa 795-1090 and western blots from human and rat pituitaries and confocal microscopy of rat pituitary sections, we have found Igsf1 protein specifically expressed in gonadotropes and thyrotropes, but not somatotropes. Our results fully agree with initial reports using double immunohistochemistry and an IGSF1 antibody made in house on the presence of Igsf1 in adult rat FSH beta +ve cells[7]. In contrast, reports in adult rat pituitary[22] and mouse embryo[4], both using an antibody made against aa 559–575 of IGSF1 showed staining in Pit-1 +ve cells and not in gonadotropes. We can only speculate about the reasons for these differences. The main ones are the use of a different antibody (different epitopes; different sources: ours commercial, others in house produced), technique (confocal versus optic microscopy) and rodent strain (Sprague-Dawley versus Wistar rats or mouse). Our pituitary data are supported by western blot, where only two bands >140 and 50 kDa are detected in rat and human pituitary. The molecular weight of the upper band is consistent with that of the main IGSF1 protein isoform (www.uniprot.org), and the lower band may represent a processed protein from plasma membrane recycling. Nevertheless, the pituitary expression pattern we describe for Igsf1 was confirmed by detection of Igsf1 mRNA in immune-purified populations from rat pituitary, an IGSF1 antibody-free methodology. While Igsf1 mRNA was undetectable in somatotropes, we confirmed our pituitary expression pattern by detecting Igsf1 mRNA in gonadotropes and thyrotropes from rat pituitary.

We then investigated *in vitro* whether IGSF1 could regulate pituitary FSH synthesis, showing that IGSF1 decreases basal transcription of a minimal human *FSHB* promoter in a dose-dependent manner. Furthermore, IGSF1 reduces Activin induction of Fshb protein expression in cultured rat gonadotropes. Finally, we showed that IGSF1 is a potent inhibitor of the Activin-Smad pathway, a route including ALK4, a type I receptor that mediates the regulatory effects of Activin on the mouse *FSHB* promoter[36].

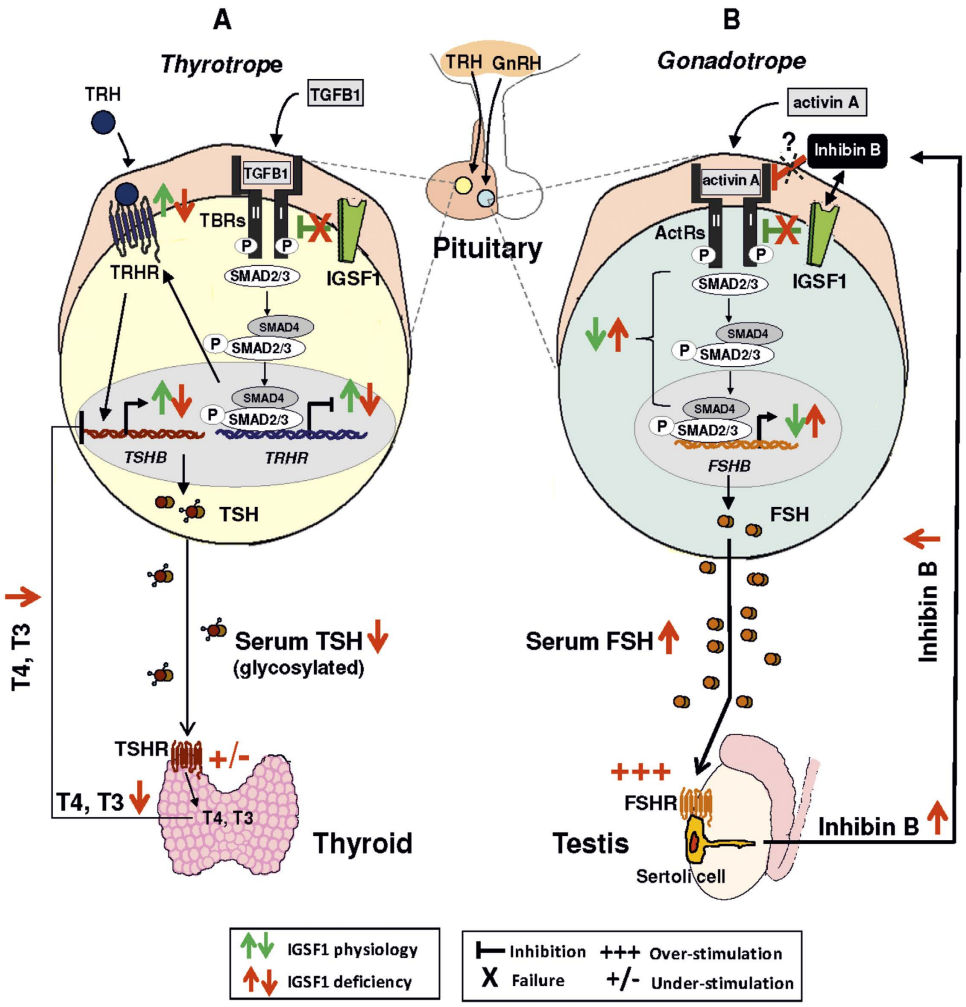


**Figure 5. Proposed model for IGSF1 functions and molecular mechanisms involved in IGSF1 deficiency.** (**A**) In thyrotropes, TGFβ1 activates TGFβ receptors (TBRs) and stimulates Smad2/3 signaling cascade leading to repression of the *TRHR* gene, which reduces the TRH-TRHR signaling over the *TSHB* expression. IGSF1 (green symbols) represses TGFβ1 pathway, positively regulating *TRHR* expression and therefore enhancing TRH-TRHR signaling, TSHB synthesis and bioactivity (glycosylation) and TSH secretion from the pituitary. In the thyroid, TSH stimulates the TSHR and the synthesis of thyroid hormones (T4, T3) which exert a negative feedback over *TSHB* expression at the pituitary. IGSF1 deficiency (red symbols) reduces TSH synthesis and bioactivity, leading to central hypothyroidism. (**B**) In gonadotropes, activin A binds activin receptors (ActRs) and stimulates Smad2/3 pathway to increase *FSHB* transcription. FSH stimulates FSHR of Sertoli cells in testis, producing inhibin B, which exerts negative feedback over pituitary *FSHB* expression. IGSF1 (green symbols) inhibits activin A pathway, negatively regulating *FSHB* expression. IGSF1 is proposed here as a mediator of inhibin B-repressive effects on *FSHB* production (black double-arrow). IGSF1 deficiency (red symbols) leads to over-secretion of pituitary FSH, inducing proliferation of Sertoli cell mass (macroorchidism) and excess synthesis of Inhibin B which, however, seems unable to exert the negative feed-back over the synthesis of FSH at the pituitary, representing a state of resistance to Inhibin B. *TRH: thyrotropin-releasing hormone*, *GnRH: gonadotropin-releasing hormone*, TGFβ*1: Transforming Growth Factor beta 1 protein*, *TBRs:* TGFβ*1 receptors*, *TRHR: thyrotropin-releasing hormone receptor*, *TSHB: thyrotropin beta subunit gene*, *TSHR: thyrotropin receptor*, *T4: thyroxine*, *T3: triiodothyronine*, *ActR: activin A receptors*, *FSHB: follicle-stimulating hormone beta*, *FSH: follicle-stimulating hormone*, *FSHR: follicle-stimulating hormone receptor*.

Both sets of results agree with a unique clinical finding in our patient with complete IGSF1 deletion: a neonatal excess of FSH secretion at "mini-puberty", when the gonadal axis is transiently active in babies[16,17]. Later in life, an aberrantly elevated FSH-secretion in response to GnRH was observed at 6 years of age, when the gonadal axis should be completely inactive. Importantly, both findings are consistent with the recent identification of increased FSH pulsatile secretion in adults with IGSF1 deficiency[48].

Taken together, our data integrate into a model for macroorchidism in IGSF1 deficiency (Fig. 5B) based on the pituitary de-repression of *FSHB* transcription at pituitary gonadotropes and the postnatal over-secretion of FSH at mini-puberty, which may lead to premature proliferation of testicular Sertoli cells[49], a known cause for macroorchidism in children and adults with FSH-secreting pituitary adenomas[25–27]. These findings are relevant

since all IGSF1-deficient patients so far identified with macroorchidism were adolescents and adults[4], which has prevented the elucidation of the precise chronology for testicular growth in this disorder. Further supports to this hypothesis are the excessive concentrations found in our patient for Inhibin and AMH, both produced by Sertoli cells. In contrast, testosterone and spermatogenesis, both dependent on Leydig-cell function , were normal. LH was not increased at any age. This clinical picture agrees with experiments in monkeys, showing that Leydig cells are absolutely dependent on LH, and that, although FSH alone could induce some Leydig cells in the interstitium, they were occasional and poorly active[50].

Sertoli cells, the larger cell type in the testis, are the main contributors to testicular size[51]. They are known to be not fully quiescent after neonatal minipuberty, but they continue to proliferate and moderately increase testicular size during infancy[52,53]. Therefore, an initially increased Sertoli cell mass at the end of mini-puberty could lead to development of macroorchidism during infancy under normal FSH levels. Alternatively, elevation of pulsatile FSH secretion could also exist in IGSF1 patients during infancy, as shown in adults with IGSF1 deficiency[48], underlying the potential to over-stimulate Sertoli cells and causing macroorchidism. Studies of FSH pulsatility require meticulous analyses with 24 h serum sampling and, so far, they have not been performed at the pediatric age[48]. Future testing of FSH pulsatility in children with the disorder may help define the early postnatal ("mini-puberty") and/or the infantile components for the timing of Sertoli cell stimulation in the IGSF1 deficiency.

We detected IGSF1 in germinal epithelium and (less) in Leydig cells in mice and human testis, but not in Sertoli cells. This suggests that the testicular phenotype is not due to primary failure of testicular IGSF1[49,54] but to overstimulation of Sertoli cells by pituitary FSH (main known stimulus for Inhibin/AMH secretion and Sertoli cell proliferation). Other researchers also found IGSF1 protein and mRNA in mouse germinal epithelium, (less) in Leydig cells and also in Sertoli cells, but only at stages VI–XIII of the maturating tubular epithelium[22]. Our human and mouse testis tissues seem to contain seminiferous tubules full of mature spermatozoids and, in those stages, our study coincides with Joustra's in finding Sertoli cells negative for IGSF1. We only found a minimal spotty double staining within the tubules between the IGSF1 +ve germinal epithelium and the Inhibin +ve Sertoli cells. It is an open question if these are regions of intimate attachment of both cell populations, unable to be solved by confocal microscopy, or if Sertoli cells are specifically +ve por IGSF1 at those spots. More studies in human testes need to be performed to clarify the contrasting staining in Sertoli cells, including a full range of ages from pre-pubertal to adulthood. *Igsf1*$^{(-/-)}$ knockout mice have normal FSH, are fertile and do not show macroorchidism[10]. Therefore, these mice are not useful to explain the increased testicular size in humans. If anything, the absence of macroorchidism in the mouse model suggests that testicular enlargement is not primarily due to the local inactivation of testicular Igsf1.

We considered the possible effect of hypothyroidism on the development of macroorchidism. This clinical association was known before the implementation of universal neonatal CH screening programs, in-pubertal boys with severe and long-standing hypothyroidism[55]. The mechanism was the cross-reactive stimulation of testicular FSHR by chronic and extremely high elevations of TSH in such boys from birth[55]. In rat models, hypothyroidism induces macroorchidism as a consequence of increased Sertoli cell numbers with reduced Leydig cell numbers at puberty[56–61]. However, two essential differences exist between the hypothyroid conditions mentioned and our patient. First, (central) hypothyroidism was congenitally present in our patient but it was readily identified and treated from birth. Second, gonadotropin levels were increased neonatally, while in hypothyroid patients (due to Hashimoto) and rat models of hypothyroidism FSH levels are strongly downregulated[56,57]. Therefore, a major derangement of pituitary gonadotropes (and not hypothyroidism) seems here to be a distinct factor involved in macroorchidism in the absence of IGSF1.

On the other hand, TRHR is expressed not only in the pituitary but also in other tissues, including the testis[62,63]. Therefore, a possible contribution of altered paracrine action of TRH on testicular TRHR in the absence of IGSF1 may deserve consideration. However, the clinical relevance of this hypothesis is questionable due to the absence of any testicular phenotype in male patients with TRHR inactivating mutations causing congenital hypothyroidism[64,65].

Finally, it is striking that the strongly elevated inhibin B levels in our patient were incapable of repressing FSH secretion, identifying a state of inhibin B resistance unrecognized in humans, and suggesting a critical role of IGSF1 as mediator of the regulation of FSH by inhibin B. Inhibin B is the major regulator of testicular-pituitary function in males through the inhibition of the Activin pathway reducing FSH synthesis and release[66]. IGSF1, formerly named *InhBP* (*inhibin binding protein*), was proposed as a possible inhibin B receptor in affinity chromatography experiments[7,8]. However, this hypothesis was ruled out after ligand-receptor binding assays could not demonstrate a direct interaction between inhibin B and IGSF1[9]. In our perspective, the failure to show direct binding between inhibin B and IGSF1 does not exclude that IGSF1 could be a "pseudo-receptor" for inhibin B. This is the case of BAMBI (BMP and Activin membrane-bound inhibitor), a membrane pseudo-receptor for different TGFβ superfamily ligands that cannot bind such ligands directly, but through the presence of type II activin receptors[67]. In light of our findings, the intrinsic molecular dialogue between IGSF1 and inhibin B in pituitary gonadotropes warrants further investigation.

In summary, we revealed here two distinct molecular effects of IGSF1 consistent with the development of central hypothyroidism and testicular enlargement in IGSF1 deficiency. IGSF1 negatively modulates two TGFβ pathways: an inhibitory TGFβ1 pathway over the *TRHR* in thyrotropes and a stimulatory activin pathway over the *FSHB* in gonadotropes. Therefore, pituitaries harboring IGSF1 defects may not synthesize enough TRHR, leading to a type of central hypothyroidism combining low TSH synthesis and bioactivity. Likewise, they may not properly repress FSH secretion, causing serum FSH elevation[48] which may lead to stimulation and proliferation of testicular Sertoli cells and macroorchidism. Thus, the major clinical features of the X-linked syndrome of IGSF1 deficiency may associate with the decreased pituitary actions of IGSF1. IGSF1 emerges as a critical regulator of different TGFβ superfamily pathways in the pituitary.

## Methods

**Hormone determinations and stimulation tests.** TSH was determined with Third Generation TSH assays on both the Immulite 2000 (Siemens Healthcare Diagnostics, Ltd, Camberley, UK) and confirmed by Vitros ECi (Ortho Clinical Diagnostics, UK) and by a two-site immunoenzymometric assay using two monoclonal antibodies (ST AIA-PACK TSH; Tosoh Corporation, Tokyo, Japan) (data not shown). Total triiodothyronine ($T_3$), total thyroxine ($T_4$) and free thyroxine ($FT_4$) in serum were measured using the Vitros ECi immunoanalyzer. FSH, LH and sex hormone-binding globulin (SHBG) were measured on the Immulite 2000 analyzer. Testosterone and estradiol levels were measured using a Coat-A-Count radioimmunoassay (Siemens Healthcare Diagnostics Ltd, Camberley, UK). α subunit was measured by an immunoradiometric assay (Immunotech SAS, France), inhibin B by Oxford Bio-Innovation ELISA (OBI-DSL Ltd., UK) and anti-müllerian hormone (AMH) by ELISA kit (Immunotech SAS, France).

For hormone assays different compounds were tested to determine specificity of the assay and none showed significant interference; cross-reactivity assays demonstrated ~100 times higher specificity for the hormone. Analytical limits of detection and dynamic range of the hormone assays are shown in Supplemental Table 1.

TRH stimulation test was performed after four weeks L-T4 treatment withdrawal. Serum TSH and prolactin were measured at −15, 0, 15, 30, 60, 90, 120, and 180 min after intravenous administration of TRH (Protirelin, 7 μg/kg body mass; max 200 μg). An inadequate TSH response was defined as a peak concentration value below 14 mIU/l and the peak/basal TSH ratio as described by van Tijn *et al.*[68]. For the study of TSH bioactivity two additional serum samples were collected at 0 and 180 min after TRH administration.

GnRH stimulation test was performed at 9 a.m. Serum FSH and LH were measured at −15, 0, 15, 30, 45 and 60 minutes after intravenous administration of GnRH (Luforan, 100 μg, Serono). Pubertal and postpubertal normal responses to GnRH were defined as described by Resende *et al.*[69].

**Mutation screening.** All coding regions of *TSHB*, the gene encoding the specific TSH beta subunit, *TRHR*, the gene encoding the TRH receptor, and *CGA*, the gene encoding the glycoprotein hormones alpha chain were amplified by PCR using appropriate primers flanking each exon. PCR products were purified and directly sequenced on an automated DNA sequencer (3100 Genetic Analyzer, Applied Biosystems).

**Comparative Genomic Hybridization Arrays (CGH Array).** High-density aCGH were performed using SurePrint G3 Human CGH Microarray 1 × 1 M (Agilent), an oligonucleotide chip that contains 963,029 distinct biological features with a probe spacing 2.1 KB overall median probe spacing (1.8 KB in Refseq genes) and Content sourced from - UCSC hg18 (NCBI Build 36).

Array experiments were performed as recommended by the manufacturer (Agilent Technologies, Santa Clara, CA, USA). 500 ng of DNA from the patient and a reference sample of the same sex (Promega, Madison, WI, USA) were double-digested using AluI and RsaI for 2 h at 37 °C. Enzymes were inactivated at 65 °C for 20 minutes and each digested sample was labeled with Cy5-dUTP by random priming at 37 °C for 2 h (Genomic DNA Enzymatic Labelling Kit Agilent). Labeled products were column-purified (Microcon Ym-30 filters, Millipore Corporation). The hybridization was performed at 65 °C with rotation for 24 h after probe denaturation and pre-annealing with Cot-1 DNA. The array was analyzed with the Agilent scanner using the Feature Extraction software (v9.1 Agilent Technologies). Comprehensive description of the statistical algorithms is available in the user's manual provided by Agilent Technologies.

**Confirmation and determination of precise deletion size.** To confirm and define the deletion size and break points, it was performed a long PCR (Expand 20Kb$^{PLUS}$ PCR System, Roche Diagnostics; Mannheim, Germany) and Sanger sequencing on the genomic DNA of the patient and parents, following manufacturer instructions. Oligonucleotide primers were designed in the flanking regions of the deletion as defined by CGH-Array taking into account the average densities of the array both in the backbone and the targeted region. For the 207,873 bp deletion in *IGSF1* gene, primers successfully determining deletion size were: Forward: 5′-gctaacccccctgtgtagtgtg-3′ and Reverse: 5′-actctgaccctcccctcct-3′.

***In vitro* TSH bioactivity assay.** TSH bioactivity of patient's sera was studied on thyrotropin receptor (TSHR)-transfected cells in luciferase reporter assays and compared with the bioactivity of recombinant human TSH (rhTSH). Human Embryonic Kidney (HEK293T) cells were stably transfected with a TSHR cDNA (generous gift from Dr. Lado-Abeal). Cells were cultured in DMEM/F12 with GlutaMax medium supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin, and 100 mg/ml streptomycin and under Zeozin (Invitrogen) treatment (200 μg/mL) to maintain the TSHR expression. HEK-TSHR were transfected with 2 μg of the pcCRE(6)-luc reporter plasmid containing 6 CRE (cyclic-AMP-Responsive-Element) promoter sites, and 1 μg of the transfection efficiency *Renilla* luciferase plasmid (pRL-SV40; Promega) in T25 flask overnight using FuGene 6 (Promega) according to the manufacturer's instructions. HEK-TSHR cells transfected with luciferase and renilla plasmids were seeded in a 96-well plate and after 8 h the growth medium was removed and stimulated cells with three different dilutions of human sera (1:2, 1:4, 1:8) and the recombinant human hormone rhTSH (Genzyme) as control in DMEM/F12 with GlutaMax medium with 0.1% BSA and pen/strep for 18 hours. After 18 h the cell-stimulating media was removed and replaced them with 25 μL of lysis buffer (25 mM Trisphosphate pH 7.8, 15% glycerol, 1% Triton X-100, 1 mM DTT and 8 mM $MgCl_2$). Luciferase assays was performed using a Dual-Glo luciferase assay system as described in the manufacturer's instructions (Promega). The firefly luciferase activity was measured adding 25 μL of luciferase reagent to lysates and analysed luminescence at a microplate luminescence counter (TopCount-Nxt tm, Packard BioScience Company). *Renilla* luciferase activity was assessed by adding an equal volume of Dual-Glo Stop & Glo substrate and measuring again in the luminometer. Firefly luciferase activities were normalized to the corresponding *Renilla* luciferase activities.

To test for unspecific responses from glycoprotein hormones in our assay, an experiment with parental HEK293 cells (without TSHR expression) transfected with pcCRE(6)-luc/pRL-SV40 was performed, showing no luciferase production in the absence of receptor (Supplemental Fig. 3) even during stimulation with the highest dose of human recombinant hormones. Further experiments showed there was no cross-reactivity in our luciferase assays between rhTSH and the FSH receptor (FSHR), nor between recombinant human (rh)FSH and the TSHR (Supplemental Fig. 3B and C).

Experiments were performed in triplicates, and repeated in three or more independent replicates. Data were represented as Relative Response Ratio (RRR), incorporating positive (pcCRE(6)-luc/pRL-SV40 transfected cells stimulated with maximal concentration of recombinant hormone) and negative control (pcCRE(6)-luc/pRL-SV40 transfected cells stimulated with the medium alone) wells within the same plate.

**Human, rat and mice tissues.** Rat pituitaries were obtained from young adult (200 g, 60 days) male Wistar rats (Animal House, University of Santiago de Compostela) and immediately frozen after dissection. Neuropituitary was discarded (rAP). The area around the marginal zone (stem cell niche) was manually dissected and discarded resulting in the majority of the adenopituitary. A single tissue microarray (TMA) was prepared with the human testes using formalin-fixed paraffin-embedded (FFPE) samples from the hospital tissue bank. Mice testicles were obtained from young adult male (60 days).

**Western blot of pituitary tissue.** Frozen pituitary fragments were mixed with 200 μL hot 1% SDS at 95 °C, homogenized in a polytron (Dremel, Racine, WI) during 10 sec and further incubated for 5 min at 95 °C in a thermoblocker. 1.5 vol of Triton Buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 10% Glicerol, 1% Triton, 5 mM EDTA, 1.5 mMMgCl, 0.1 M PMSF, 5 mg/ml Aprotinin, 2% Na3VO4, 0.1 M NaPyrophosphate) was added and sample homogenized through a 25 g syringe followed by an incubation in ice for 20 min. Eppendorf tubes were centrifuged at 14000 rpm for 5 min at 4 °C. The supernatant was kept at −80 °C. 75 μg of proteins were loaded per lane.

**Immune Purification of secretory cell populations with magnetic beads and qRT-PCR quantification.** The immune purification of secretory cell populations was established in our lab previously[70]. We have modified the protocol for RNA quantification as described[71]. Briefly, six pituitaries were dissected and dispersed to single cells with collagenase and DNAse for 20 minutes and washed three times with DMEM. Single-cell dispersions were fixed by adding 1 vol ethanol 100% (final concentration: 50%) for 15 minutes in ice. Cells were washed three times with PBS, divided into five tubes, three for incubation with the respective anti-hormone antibody (see Supplemental Table 2), one for non-antibody control beads purification and one for direct extraction as control for single-cell dispersion. Tubes were kept for 30 minutes in ice. After washing with MACS buffer (Miltenyi), cells were incubated with 1:4 magnetic beads coupled to anti-rabbit antibody (MACS, Miltenyi) for 15 minutes in ice. Cells were washed again once with MACS buffer and added to the column attached to the magnetic separator (Miltenyi).

RNA was extracted with Trizol, resuspended in 12 microliters of water and incubated with 1unit of RNAse-free DNAse (Thermo) for 30 min at 37 °C, followed by inactivation. Reverse-transcription was performed in 30 μl using 250 units of MMLV (Invitrogen). Quantitative PCR was performed with 1 μl of the reaction with SybrGreen (Brilliant III Ultra-fast, Agilent) in an Abyprism 7500 (Applied Biosystems) using the following primers: rattus-Gh-F-TGGCTGCTGACACCTACAAAGAG; rattus-Gh-R-CCTGGGCATTCTGAATGGAA rattus-Fshb-F-GATAGCCAACTGCACAGGACATAG; rattus-Fshb-R-ATGCAAAGCTGGATCGACTTC; rattus-Tshb-F-TCTGCGCTGGGTATTGTATGAC; rattus-Tshb-R-CAGACATCCTGAGAGAGTGCGTACT; rattus-Igsf1 (primers designed on the epitope recognized by the commercial antibody used in this wor k)-F-CAGCCTAGCAACACTCTGGAACT; rattus Igsf1-R-TGGACTAACAGGCTGGGCTTA; rattus-18s-F-5′ CGGCTACCACATCCAAGGAA3′; rattus-18s-R-5′GCTGGAATTACCGCGGCT3′. For quantitative mRNA expression, relative ΔCt values for every gene were referred to its Tbp ΔCt in every sample, and the average of the ΔCt signal in the control unpurified cell dispersion was taken as 1 (ΔΔCt) for every gene. Experiments were performed in quadruplicate.

**Inmunohistochemistry of testicular tissue.** Immunohistochemical studies were performed in paraffin section of adult human testes with anti-IGSF1 rabbit polyclonal antibody (GeneTex) and different testicular antibodies (Supplemental Table 2). Sections were stained using an automated system DAKO Austostainer. Antigens were retrieved with the Dako buffer citrate pH 6 in a DAKO PT Link for IGSF1 and pH 9 for inhibin for 60 min. Immunohistochemistry was performed using the Dako Envision Flex kit. Immune reactions were developed with diaminobenzidine, and sections were counterstained with Harris' hematoxylin. As negative controls, adjacent sections were subjected to the same immunohistochemical procedure omitting the primary antibody.

**Immunofluorescence and confocal co-localization.** Tissues were fixed in neutral formalin and included in paraffin as described[70,72]. 3 μm sections were mounted in superfrost coated slides (Thermo) and directly processed for automatic antigen retrieval in a PT-link (DAKO) using the pre-programmed cycle at 97 °C followed by blocking in 50 mM NH4Cl during 20 minutes. Antibody incubations (Supplemental Table 2) were intermixed with washing in PBS. Finally samples were mounted in Fluorogel (Electron Microscopy) with 2 μg/ml DAPI (Sigma). Negative controls were performed in the absence of both primary antibodies, or with one primary but not the other, always followed by both secondary antibodies and DAPI. Microscopy analysis was performed in a TCS SP5 X white light laser including a UV laser configuration (Leica) by exciting at 405, 492 and 552 nm while capturing maximal emission at 414–479, 498–547 and 565–624 nm sequentially between frames. Whole Sections were recorded first at low magnification (20x) in three pictures (right lobe, centre and left lobe); thereafter at least 6–10 fields per area were recorded at 63x followed by zooming in (z4) for some specific cell groups. Negative controls without 1st antibody did not show any intense signal able to be recorded at 498–547 and 565–624, but

presented intense staining at 419–479 (DAPI); negative control omitting IGSF1 antibody presented a strong signal for hormone (Supplemental Fig. 7).

Quantifications were performed using ImageJ/Fiji (Rasband, W.S., Image J, U. S. National Institutes of Health, Bethesda, Maryland, USA,http://imagej.nih.gov/ij/, 1997–2012). Around 400–500 cells were counted per picture in at least six independent pictures from three different pituitaries.

**In silico characterization of TRHR and FSHB promoters.** To identify potential Smad binding elements (SBEs) in the *TRHR* and *FSHB* promoters, the Multi-genome Analysis of Positions and Patterns of Elements of Regulation (MAPPER2, http://genome.ufl.edu/mapper/mapper-main) program was used. MAPPER2 is a platform for the computational identification of transcription factor binding sites (TFBSs) in multiple genomes that combines the use of TRANSFAC and JASPAR databases. The platform and databases include Smad3 (TAGCAGACAG) and Smad4 (GTGGGGCAGCCACT) consensus sequences.

**Expression plasmids, reporter constructs and reagents.** The expression vector for human pCMV-SPORT6.1-IGSF1 was purchased from Cultek. Human pGL2-pTRHR luciferase reporter vector constructed from −2530 to +1 (initiator ATG position) has been described in Matre *et al.*[34]. The human *FSHB* promoter was PCR-amplified in a 995 bp product comprising the sequence −875 to +120 relative to the transcription start site (NM_000510) using 5′-gctgttgtccttttgctccagtc-3′ as forward primer and 5′-gctctagttttgtcctccatgtcc-3′ as reverse primer, and cloned into the pGL3 basic vector (Promega). Artificial pGL3-pCAGA luciferase reporter vector containing Smad3 binding elements was provided by Dr. Jenny Visser (Erasmus MC, Rotterdam, The Netherlands) as was constitutive active pCDNA3.1-ALK4 type I activin receptor-like kinase (caALK4). Dominant-negative mutant of Smad2 into pCDNA 3.1 (DSmad2) was provided by Dr. Carmelo Bernabéu (Biological Research Center, CIB, Madrid, Spain).

Recombinant activin A (R&D Systems, Minneapolis, MN) was dissolved in PBS/0.1% BSA and used at 2–80 ng/ml. SB431542, TGFβ pathways inhibitor (Selleck Chemicals), was dissolved at 10 mM in DMSO and used at 5 μM/ml final concentration. TGFβ1 (Calbiochem, Merck-Millipore) was dissolved in 4 mM HCl-0.1%BSA and used at a range of concentrations 0.125–5 ng/ml. When not indicated, it was used at 2.5 ng/ml.

**Transient transfections and luciferase assays.** HEK293 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS, Gibco), 100 IU/ml penicillin, and 100 mg/ml streptomycin at 37 °C in 5% $CO_2$. One day before transfection, cells were seeded in 12-well culture plate. At 50% confluency, HEK293FT cells were transfected with FuGene 6 transfection reagent (Promega) using 500ng of DNA per well or increasing concentrations of IGSF1 plasmid (0, 50, 100, 150, 200 ng/well) and a reporter vector in constant doses (300 ng/well), for dose-response curve experiments; and with 250ng total DNA per well with ratio 1:3 between the expression plasmid and the reporter vector, for activin pathway experiments, or with kit V-program A023 (Amaxa) using 1.25 mg total DNA per cuvette distributed in 0.5 mg human pTRHR-luc or pCAGA-luc, 0.5 mg IGSF1 or Empty-CMV, and 0.5 mg pCMV-bGal. 24 hours after transfection, cells were treated with recombinant protein activin A with/without SB431542 or vehicle treatment in DMEM with 0.2% FBS and pen/strep for 24 hours.

To test TGFβ pathway, HEK293FT cells were transfected with 500 ng of total DNA per well: 200 ng of IGSF1 plasmid or pcDNA and 300 ng of pTRHR reporter vector. 24 hours after transfection, cells were treated with recombinant TGFβ1 with/without SB431542 or vehicle treatment in DMEM with 0.2% FBS and pen/strep for 24 hours. In all cases co-transfection with CMV-renilla or CMV-β-gal reporter plasmid was performed. Dual-luciferase reporter assay (Promega) was accomplished as described in the manufacturer's instructions (Promega), and measuring using a luminometer Glomax 96 microplate (Promega). Experiments were performed in quadriplicates, and repeated in three or more independent replicates.

**Transient transfection, real-time quantitative PCR and western blot in pituitary cells.** GH4C1 cells were cultured as described previously[72]. Three days later, cell were transfected with 3 μg of IGSF1 or empty vector (pcDNA3) plasmid/well in 12-well culture plates. Transfection was performed by Nuclefection (Amaxa, Lonza) using the A020 program as described[70,72]. 30 hours after transfection, cells were treated with TGFβ1 for 24 hours. RC-4B/C1 were cultured as described[38] except that DMEM was 1gr/l glucose. They were transfected with 0.5 μg DNA/well Turbofect (Thermofisher) for 24 hours.

RNA was extracted with Trizol. 1 μg of total RNA was incubated with 1unit of RNAse-free DNAse (Thermo) and reverse-transcribed as above. Quantitative PCR was performed with 1 μl of the reaction using the following primers: Trhr-F-cagatgtttcaacagcaccg; rattus-Trhr-R-ggttgtaaatcaccgggttg; rattus-Tbp-F-cttcgtgccagaaatgctgaa; rattus-Tbp-R-cagttgttcgtggctctcttattctc. For quantitative mRNA expression studies, relative ΔCt values for Trhr were referred to its Tbp ΔCt in every sample, and the average of empty vector/vehicle-treated controls was taken as 1 (ΔΔCt) for all groups. Experiments were performed in triplicates and independently repeated three times.

Proteins were extracted and phospho-SMAD2/SMAD2/3 and total Smad signaling and was performed as described[41] and explained in Supplemental Table 2, where conditions for Fshb are included.

**Statistical analysis.** Statistical significance was defined as $P < 0.05$ and was determined by two-tailed *t* test for parametric samples after assessing that samples did follow a normal distribution with the two-samples Kolmogorov-Smirnov test (Graphpad Prism v5.0).

**Ethic statement for human studies.** Human pituitary sample extracts (frozen material) and normal human testes were obtained from the Biobank of the Department of Pathology, Santiago de Compostela University Hospital (Spain), registered as GAL, n° 52 within the approved Biobank Network of the Spanish Ministry of Health (ISCIII, http://www.redbiobancos.es/Pages/Docs/triptico_donantes_A5_cerrado_141031.pdf).

All experiments were performed in accordance with relevant guidelines and regulations. All experimental protocols were approved by the Biobank Committee of the Santiago de Compostela University Hospital (Spain). Written informed consent was obtained from all subjects previous to surgery.

**Ethic statement for animal experiments.** Animal experiments were performed in accordance to the license 15010/14/005 granted to CV Alvarez. All experimental protocols were approved by the official licensing Committee ("Xefatura Territorial da Conselleria do Medio Rural e do Mar") of the Regional Government of Galicia (Spain). This Committee guarantees that animal experimentation is performed attending the ethic guidelines required by European and National legislation (RD53/2013).

**Informed consent.** Informed consent for genetic studies was obtained from the index patient and his parents, according to protocols followed at the Ramón y Cajal University Hospital (Madrid, Spain), where the patient was clinically followed.

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## Author Contributions

J.C.M., R.B. and C.V.A. conceived the study. R.B. clinically diagnosed the patient. J.C.M., M.G.L., C.V.A., J.M.C.T., M.N. and M.P.M. designed experiments. Experiments were performed by M.G. with important contributions of M.G.L. and A.R.G.R.; also performed experiments E.D.R., A.E., D.G.D.B., A.F. and E.V. Y.B.d.R. was involved in data collection and interpretation. A.R.G.R., J.C.M., M.G., C.V.A., J.M.C.T., M.N., M.P.M., A.C.S.H.K., J.N., P.L., V.M., P.M.H. analyzed and interpreted the data. J.C.M., C.V.A. and M.G. wrote the manuscript, which was reviewed by all authors.

## Additional Information

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# CAPÍTULO III

# Hipotiroidismo congénito con disgenesia tiroidea y macroorquidismo en dos hermanos con deleción en IGSF1

IGSF1 es una proteína de membrana con 12 dominios inmunoglobulina, codificada en el cromosoma X y expresada en hipófisis y testículo. Mutaciones puntuales, pequeñas deleciones y deleciones completas del gen causan hipotiroidismo congénito central (HCC) y macrooquidismo. Sin embargo, existe una variabilidad fenotípica interindividual en el tamaño tiroideo de causa desconocida.

Los objetivos de este trabajo se centran en el estudio genético del "Síndrome IGSF1" en dos hermanos con HCC y macroorquidismo asociado a severa hipoplasia tiroidea.

Para ello se realizó un seguimiento hormonal del eje hipófisis-tiroides y una gammagrafía tiroidea a los pacientes. En un panel de secuenciación masiva (NGS, NextSeq 500) se estudiaron 390 genes de patología tiroidea. MLPA (P319-A2-Thyroid) para descartar CNVs en genes de hipoplasia tiroidea (TSHR, PAX8, FOXE1, NKX2-1). RT-PCR e inmunohistoquímica de IGSF1 en tiroides.

Dos hermanos (26 y 20 años) fueron diagnosticados de HCC (T4: 4/0,44pmol/L, N: 11-25pmol/L; TSH normal: 2,7/2,8mU/L; N: 1,5-5,7mIU/L) en los primeros días de vida, iniciando tratamiento sustitutivo (L-T4). A los 3 años son reevaluados con suspensión de L-T4 por un mes, mostrando ambos un hipotiroidismo con TSH muy elevada (48/81,8 mU/L) y disgenesia tiroidea severa, típicos de hipotiroidismo primario. Por NGS y Sanger se identificó una duplicación de 7pb seguida de una deleción de 739pb en la parte carboxi-terminal del gen *IGSF1* [c.3488-59_3775delinsCAATAAG] que reordena los exones 17 al 19. NGS y MLPA descartan defectos genéticos en genes de hipoplasia tiroidea. El cDNA y proteína de IGSF1 se expresan abundantemente en tiroides.

Por primera vez se identifica un reordenamiento complejo tipo INDEL en *IGSF1,* asociado a un hipotiroidismo mixto, central y primario. Sin poder descartar defectos en genes del desarrollo tiroideo desconocidos hasta la fecha, la inédita y abundante expresión tiroidea de IGSF1 implica la posible participación directa de este defecto en el desarrollo embrionario del tiroides por interferencia de rutas TFGB y Activina en las que participa demostradamente en células tirotropas de la hipófisis.

# Congenital central hypothyroidism with severe thyroid hypoplasia in two brothers with IGSF1 intragenic deletion

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## Abstract

IGSF1 is a membrane protein with 12 immunoglobulin domains, encoded from the X chromosome and expressed in pituitary and testis. Point mutations, small deletions and complete deletions of the gene cause central congenital hypothyroidism (CCH) and macroorchidism. However, phenotypic variability is observed in other endocrine features, including the unexplained decreased size of the thyroid gland.

**Objective:** Genetic investigation of the "*IGSF1* Syndrome" in two brothers with CCH and macroorchidism associated with severe thyroid hypoplasia.

**Patients and Methods:** Detailed characterization and hormonal follow-up of the pituitary-thyroid axis in two brothers with CCH. Targeted next generation sequencing (NGS, NextSeq 500) of 390 thyroid genes. MLPA of *TSHR, PAX8, FOXE1* and *NKX2.1* genes and genome-wide dense SNP array (CytoSNP-850K) for detection of copy number variations (CNV) in candidates for human thyroid dysgenesis. RT-PCR and immunohistochemistry of IGSF1 from human thyroid tissue.

**Results:** Two siblings, now 26 and 20 years of age, were diagnosed at birth with CCH in a T4-based congenital hypothyroidism screening program in Cyprus. They showed T4 levels of 4 and 0.44 pmol/L (N: 11-25 pmol/L) and TSH of 2.7 and 2.8 mU/L (N: 1.5-5.7 mIU/L), respectively, and were started with substitutive levo-thyroxine (L-T4). At 3 years of age, they were re-evaluated after L-T4 withdrawal, both showing very high TSH (48/81.8 mU/L) and severe thyroid dysgenesis at thyroid scintigraphy, typical of primary hypothyroidism. NGS and Sanger sequencing identified a duplication of 7bp followed by a deletion of 739bp at the carboxy-terminus of *IGSF1* [c.3488-59_3775delinsCAATAAG] rearranging exons 17 to 19. NGS, MLPA or SNP-array could identify defects in genes known to be associated with human thyroid hypoplasia. IGSF1 cDNA and protein were shown to be abundantly expressed in the human thyroid tissue.

**Conclusions:** For the first time, a complex INDEL rearrangement is identified in the *IGSF1* gene, associated with “mixed phenotype” of central (pituitary) and primary (thyroid) hypothyroidism. The absence of common mutations in genes for human thyroid hypoplasia and the previously unreported presence of *IGSF1* in thyroid, may suggest possible involvement of IGSF1 in development and maturation of the thyroid gland.

## Introduction

Central congenital hypothyroidism (CCH) is caused by a deficient production and bioactivity of TSH and low thyroid hormones synthesis from an otherwise normal thyroid gland (García M *et al.*, 2014, Annex I of this Thesis). The TSH-based neonatal screening programs implemented in the most European countries safely detect primary hypothyroidism but congenital hypothyroidism (CH) cases of central origin are not detected, leaving this group of entities undiagnosed. However, uncommon T4-based CH screening programs in few countries detect higher some CCH cases, estimating the prevalence of central CH as of 1 in 16,000-30,000 (van Tijn DA *et al.,* 2005; Adachi M *et al.*, 2012).

Given the physiological complexity of the central regulation of thyroid hormone synthesis, molecular bases of central CH are largely unknown. Nowadays, genetic defects in only 4 genes have been identified in patients with isolated CH: *TSHB* (encoding the B-subunit of the TSH glycoprotein hormone), *TRHR* (the specific 7-transmembrane domain receptor for hypothalamic TRH), *IGSF1* (a protein regulating the expression of TRHR in pituitary thyrotropes), and the recently identified *TBL1X* (transducing β-like protein 1, X-linked) (García M *et al.*, 2014; Heinen CA *et al.*, 2016, Annex I of this Thesis).

IGSF1 is a membrane protein with twelve C2-type immunoglobulin (Ig) loops, similar to a kinase membrane receptor but devoid of any kinase activity. Missense, nonsense and frameshift mutations, disrupting the extracellular domain of the protein, and complete deletions of the gene have been described in patients with an X-linked syndrome of CCH as main feature (Sun Y *et al.*, 2012). Moreover, a variable proportion of affected males present other features like macroorchidism, hypoprolactinemia, (transient, partial) growth hormone deficiency, delayed puberty or increased body weight (Joustra SD *et al.*, 2016A). This X-linked syndrome is more severe in males, but female cases are described with milder central hypothyroidism but normal growth and pubertal development (Joustra SD *et al.*, 2013). Only recently, the molecular mechanisms underlying two most prominent features of the syndrome were unraveled (García *et al.*, 2017A; Turgeon MO *et al.*, 2017). But we still need to deepen into the function(s) of IGSF1 in cell types other that pituitary thyrotropes and gonadotropes in order to understand other features of the syndrome appearing with unexplained variability.

Central congenital hypothyroidism in the IGSF1 syndrome is of pituitary origin, and presents with decreased TSH bioactivity and TSH secretion at the TRH stimulation test (García *et al.*, 2017A). Similarly, macroorchidism is a peripheral feature of a pituitary defect since neonates show FSH elevation at minipuberty and adult males show increased FSH pulsatile secretion

(García *et al.*, 2017A; Joustra SD *et al.*, 2016B). This is consistent with the predominant expression of IGSF1 in pituitary (Chong H *et al.*, 2000). Recently, many patients have identified with a small thyroid gland below 2.5$^{th}$ percentile and even with unmeasurably small gland in some patients. While this could be attributed to the lack of trophic stimulation of thyroid, either by low TSH bioactivity or by the life-long suppression of TSH in patients diagnosed and treated with L-T4 from birth, the possible expression and function of IGSF1 in the thyroid gland itself remains to be elucidated.

Here we described a novel phenotype in two brothers with congenital hypothyroidism of central origin diagnosed and treated in the first days of life, but presenting unexpected elevations of TSH and signs of severe thyroid dysgenesis after L-T4 treatment withdrawal in two separate re-evaluation of their thyroid axes. At follow-up, CCH was associated with clear macroorchidism, both phenotypes consistent with the identification of a complex rearrangement in the *IGSF1* gene. For the first time, *IGSF1* defects are associated with hyperthyrotropinemia. Moreover, we show here for the first time that IGSF1 mRNA and protein are expressed in the human thyroid gland, opening avenues for novel research towards a more complete understanding of the role of IGSF1 in the hypothalamic-pituitary thyroid hormone axis.

## Methods

### *Hormonal determinations*

CH screening in Cyprus started in 1990. Initially total thyroxine (T4) and TSH were determined, but as from 1992 only the TSH is measured using a solid phase assay. The TSH is measured with the two-site fluoroimmumometric assay using the DELFIA® Neonatal hTSH kit (Wallac Oy, Turku) by quantitatively determining thyrotropin (TSH) levels in duplicate blood specimens. The assay procedure was followed as outlined in the kit insert with an overnight incubation period at 4 ºC. The fluorescence in each well was then measured by the 1232 Delfia fluorometer at a wavelength of 340nm. TSH concentrations were calculated automatically by the Multicalc software in µU/ml against the standard curve. All values above 12 µU/ml but below 25 µU/ml were rescreened by the same method using duplicate blood spots from a second card. If the TSH concentration from the first blood spot analysis was above 25 µU/ml or the second blood spot analysis above 12 µU/ml the infants were recalled to give a sample for further quantitative measurements of their serum Total thyroxine (T4), and TSH levels. DPC's

Coat-a-Count Total T4 (a radioimmunoassay) and Coat-a-Count TSH IRMA (Immuno-radiometric assay) kits were used according to manufacturers' instructions.

***Mutation screening***

Informed consent for genetic studies was obtained from index patients and their parents, according to protocols followed at the Makarios Hospital (Nicosia, Cyprus) where patients are clinically followed.

The complete coding regions of *TSHR* (TSH receptor), *PAX8* (member of the paired box family of transcription factors), *TSHB* (specific TSH beta subunit), *TRHR* (TRH receptor) and *IGSF1* (immunoglobulin superfamily factor 1) genes were amplified by PCR using appropriate primers flanking each exon. PCR products were purified and directly sequenced on an automated DNA sequencer (3100 Genetic Analyzer, Applied Biosystems).

A targeted panel including the coding sequence and flanking intronic regions of 390 genes implicated in thyroid pathophysiology in human, animal and cell models were studied by Next Generation Sequencing (NGS) with NextSeq 500 (Illumina) platform. Variants were analysed following strict criteria for population frequency (<1% in public variant dadabases) and *in silico* prediction of pathogenicity (García *et al.*, 2017B).

***Confirmation and determination of precise IGSF1 deletion size***

To confirm and precisely define the *IGSF1* deletion identified, we designed PCR-specific oligonucleotide primers flanking the region of the deletion identified by NGS, which initially resulted in the absence of amplification of exons 17, 18 and 19 of the gene. Such primers allowed differentiation between homozygotes and heterozygotes for the deletion and non-carrier subjects, based on the different size of the PCR fragments amplified from genomic DNA of each subject. Amplified DNA fragments from homozygotes and heterozygotes were sequenced to exactly determine the size and break points of the deletion. From the series of upstream and downstream set of primers designed for such aim, the flanking regions of this 739 bp deletion were efficiently amplified and sequences using the following primer pair: *Forward:* 5'-TGACTCTTCAGTGTCAAGGG-3' and *Reverse:* 5'-AAACAGATTGCACGGTGGG-3'.

**MLPA**

Multiplex ligation-dependent probe amplification (MLPA) using the commercial SALSA MLPA probemix P319-A2 Thyroid (MRC-Holland, Amsterdam, The Netherlands) was performed to detect deletions and duplications in *TPO, PAX8, FOXE1, NKX2.1* and *TSHR* genes related to human thyroid dysgenesis and analyzed using Coffalyser software.

**CGH-SNP-Array**

Comparative Genomic Hybridization Array (CGH Array) was performed using CytoSNP-850K BeadChip (Illumina), containing 850,000 selected single nucleotide polymorphisms spanning across the genome. This high density of SNPs enables high-resolution analysis for discovery of significant chromosomal aberrations.

Array experiments were performed as recommended by the manufacturer (Illumina, San Diego, California, USA).

***IGSF1 amplification in Human Thyroid Gland cDNA***

To determine the expression of *IGSF1* in thyroid tissue, a PCR from Human Thyroid Gland Marathon-Ready cDNA template (Takara, California, USA) was performed following manufacturer´s indications, using specific exonic primers for the human *IGSF1* gene. *IGSF1* primer sequences were: *Exon2-forward:* 5'-GCTACTGGAAGGAGACAGGCT-3' and *Exon3-reverse*: 5'-GTTGCTGGGCTCTGACCACA-3'. Amplified fragments were visualized in 2% agarose gels. Human Testis Marathon-Ready cDNA (Takara, California, USA) and genomic DNA from a healthy individual were used as amplification controls.

***Immunohistochemistry of the thyroid gland***

Formalin-fixed paraffin-embedded human thyroid sections were mounted on xilano-covered glass slides, dried overnight at 45ºC, de-paraffinized, and hydrated. Antigen retrieval was performed by boiling the slides immersed in citrate buffer with pH=6 for 90 secs. The tissues were washed in phosphate buffer saline (PBS). Blocking of non-specific binding sites was performed by incubation in goat pre-immune serum diluted 1:20 in PBS containing 1% BSA (PBS-BSA) for 30 min at room temperature (RT). Incubation with primary anti-IGSF1 antibody (GeneTex, GTX112633) diluted 1:200 in blocking solution was carried out at 4ºC overnight, followed by washing in PBS. Bound antibodies were visualized through inclubation for 1 hour with 1:100 diluted anti-rabbit biotin (Vector) at room temperature, and ABC-AP kit (Vector). Cell nuclei were counterstained with hematoxylin.

## Results

***Clinical Cases***

The first infant (Patient 1) of healthy non-consanguineous parents was born by induced normal delivery with 4.1 Kg birth weight (BW) without clinical symptoms of hypothyroidism (Figure 1A). He developed jaundice on the 2$^{nd}$ day of life and required phototherapy for 48 hours. He

presented low total T4 (2.5 µg/dl; N: 5-15 µg/dl) but not elevated (actually undetectable) TSH (0 µIU/ml, N.R.: <12) on the congenital hypothyroidism screening test. Extensive evaluation revealed low levels of total T4 and T3 and low free T4 and T3, whereas repeated TSH measurements were all within normal range (Table 1). The thyroxin binding globulin (TBG) levels were normal (16 mg/l: N: 7-17 mg/l), and thus TBG deficiency was excluded. The baby was started on L-T4 treatment on day 48 (15 µg/Kg/day). Evaluation of his hypothalamic-pituitary function did not reveal further abnormalities. At the age of 3 months, 24h cortisol profile was normal. At 6 years of age, basal prolactin (150 mIU/L) and IGF-1 (125 ng/ml) were in the normal range. The patient showed delayed bone age from 8 to 22 months of life but he grew normally and presented mild overweigh (BMI: 25.24) at 26 years of age. At 1 year of age, patient showed unilateral macroorchidism which evolved to bilateral increased testicular size at 3 years of age (4 ml) (Table 1, Supplemental Figure 1) (Joustra SD *et al.*, 2015). At this age, reevaluation of CH was treatment was withdrawn showing hypothyroidism with elevated TSH (48 µIU/ml) and an eutopic but hypoplastic thyroid gland but at thyroid scintigraphy (Table 1, Figure 1B). At the age of five years treatment was again withdrawn, and similar hormonal findings recurred (TSH: 140 µIU/ml) (Figure 1C, D). The patient started his puberty in a timely way, at the age of 12 years, with yet enlarged testicular volume (12/15 ml, p98), as measured by orchidometer (Table 1, Supplemental Figure 1).

His brother (Patient 2) was born six years later with normal delivery and BW of 3.7 Kg. On the 2$^{nd}$ day of life he developed severe jaundice and required total blood exchange. On congenital hypothyroidism screening only TSH was measured which was low (0.2 µIU/ml). After screening he showed hormonal determinations overlapping other of his brother´s: central hypothyroidism with very low FT4 (0.44 pmol/l, N: 11-25 pmol/l) and normal TSH (2.78 µIU/ml, N.R.: 1.5-5.7 µIU/ml) (Table 1). He was also started on L-T4 on day 15 (at 15 µg/Kg/day). At 1 year of age, he developed mild bilateral testicular enlargement (3/3 ml), always around percentile 98 (Table 1, Supplemental Figure 2). After discontinuation of the treatment, at the age of 3 years, TSH raised and the thyroid scan revealed apparent agenesis of the thyroid gland (Figure 1B). While on treatment, his TSH level was fully suppressed, typical of central hypothyroidism, like his brother´s TSH (Table 1, Figure 1C, D). Alterations in pituitary function were excluded with basal PRL (180 mIU/L) and IGF1 (220 ng/ml) within normal range at seven years of age. He showed normal growth but also developed overweight (BMI: 27.78) at the age of 20 years). The boy started his puberty at normal age, around 12 years, with testicular enlargement (15 ml, p98) (Table 1, Supplemental Figure 2).

***Deletion and duplication in IGSF1 gene***

Molecular analysis for mutations by Sanger sequencing of 4 candidate genes associated with thyroid dysgenesis (*TSHR* and *PAX8*) and central hypothyroidism (*TSHB* and *TRHR*) did not reveal defects in any of the 2 patients. Targeted-NGS of 390 genes associated with thyroid pathology excluded the presence of pathogenic variants related to the disease in the patient 2.

MLPA excluded copy number variations (CNVs) in the main genes related with thyroid dysgenesis (TPO, PAX8, FOXE1, NKX2.1and TSHR) in parents and patient 2 (Supplemental Figure 3).

PCR from genomic DNA of both siblings failed to amplify exons 17 to 19 of the *IGSF1* gene, whereas they were amplificable from DNA of their parents (Figure 1E). To confirm and defined the break-point of the deletion in the *IGSF1* gene of patients, PCR and Sanger sequencing were performed with specific primers flanking exons 17 and 19. DNA of both patients and their mother amplified in a short fragment around 600 bp, whereas the DNA of the father amplified in a long fragment around 1400 bp, revealing that the defect is inherited from the mother, who carries it in the heterozygote stage, whereas it is absent in the father (Figure 1F). Sanger sequencing showed in both siblings a hemizygous duplication of seven base pairs (CAATAAG) in intron 17 followed by a deletion of 739 bp, which includes part of intron 17, the complete sequence of exon 18, the entire intron 18 and the 9 most 5´-located basepairs of exon 19 of the *IGSF1* gene (Figure 2). The internationally recommended nomenclature for this complex defect is: c.[3487+56_+62dup;3488-59_3775del] or c.[3488-59_3775delinsCAATAAG] (Figure 2).

This indel rearrangement is predicted deleterious for the structure of the IGSF1 protein and its function since the defect is located it the twelfth Ig loop, upstream of the transmembrane domain, probably disrupting the anchoring of the protein to the membrane.

***IGSF1 is expressed in human thyroid gland***

In order to determine the expression of IGSF1 in human thyroid, we performed a RT-PCR using commercial cDNA template generated from a pool of human thyroid glands donors. We demonstrate that IGSF1 mRNA is present in human thyroid glands as well as in human testis, used as amplification control (García *et al.*, 2017A). The agarose gel showed a fragment of 325 bp amplified from both thyroid and testis cDNAs, which corresponds with the mRNA post-splicing between exons 2 and 3 of the gene (Figure 3A). The amplification of a fragment of 625 bp from genomic control DNA but not from thyroid and testis cDNA, excludes genomic contamination of used cDNAs (Figure 3A).

To determine the presence of the protein we performed immunohistochemistry of human thyroid gland. We demonstrate that IGSF1 protein is present in the thyrocytes of follicles showing the most thickened epithelium (taller cells) which are assimilated to those with more active synthesis of thyroid hormone (Figure 3B, C). The pattern of expression in the follicular cell is cytoplasmic and granular, with reinforcement at the apical border (Figure 3D, E).

## Discussion

CCH is an underdiagnosed group of disorders in most countries worldwide. Only few T4-based neonatal screening programs detect substantial numbers of neonatal CCH. For this reason, the first patients reported with *IGSF1* defects were detected in The Netherlands and Japan, both countries implementing sophisticated T4-based screening programs respectively using total T4/TSH/TBG strategies or the determination of Free T4/TSH -in some Japanese prefectures- from filter paper blood spot (van Tijn DA *et al.*, 2005; Adachi M *et al.*, 2012). As screened by such programs, babies with *IGSF1* defects showed low (or borderline-low) T4 and normal TSH (Joustra SD *et al.*, 2013; Joustra SD *et al.*, 2016A).

Consistently, our patient 1 was detected at a 2-year trial program (1990-1992) using T4-based strategy in Cyprus, showing low total T4 but not elevated TSH. However, from 1992 only TSH is measured, and his brother, patient 2, would have escaped screening if his brother had not had been previously diagnosed with central hypothyroidism. Patient 2 showed normal TSH at screening but he was reevaluated at 15 days of life, showing very low serum FT4. Nowadays, as in Cyprus, most European countries, do not routinely detect neonatal CCH cases. Moreover, both brothers presented testicular enlargement from infancy.

The pathogenic molecular mechanisms mediating the core phenotypes of the "IGSF1 syndrome", central hypothyroidism and macroorchidism, remained obscure until recently (García M *et al.*, 2017A). Now, the role of IGSF1 in pituitary thyrotropes and gonadotropes as modulator of Activin- and TGFB1-mediated signalling pathways was revealed, being involved in transcription of *TRHR* and transcriptional repression of *FSHB*, respectively.

However, the observation of large phenotypical variabilities among patients is a well-established phenomenon in the human syndrome (Joustra SD *et al.*, 2013; Tajima T *et al.*, 2013; Joustra SD *et al.*, 2016A). Prolactin and (partial) GH deficiencies or obesity have been less consistently reported in IGSF1 patients. This leaves expression and function of IGSF1 in different other cells types in the open, representing an extensive field for research. Recently, a

substantial decrease in the size of the thyroid in *IGSF1*-deficient patients was reported, in children and adults (Joustra SD *et al.*, 2016A).

At 3 years of age, our patients were re-evaluated for confirmation of permanent CH and for etiological investigations of the disorder, including thyroid scintigraphy. Unexpectedly for documented central CH cases, both brothers showed striking elevations of TSH at levels characteristic of primary CH. Thyroid isotopic uptake indicated severe reduction of the size or absence of thyroid tissue and activity (Figure 1B). The same pattern of marked hyperthyrotropinemia repeated after a second T4-withdrawal re-evaluation at 5 years of age in both siblings (Figure 1C).

Therefore, this is the first report of IGSF1 patients showing highly elevated TSH (>150 mIU/L) associated with severe thyroid dysgenesis in the re-evaluation for the thyroid axis during infancy.

The mechanism of this unreported feature warrants further investigation. Here we consistently excluded mutations or CNVs in all known genes causing human thyroid dysgenesis, by means of three different and complementary genetic and genomic techniques. Beside the possibility of this thyroid hypoplasia in these patients being caused by defects in a yet unidentified gene, we explored the chance that the IGSF1 defect itself could be involved in thyroid morphogenesis. Using RT-PCR and immunohistochemistry we show here for the first time that IGSF1 mRNA and protein are abundantly expressed in human thyroid tissue. This supports the hypothesis that IGSF1 could be not only involved in the throphic effect of TSH at the thyroid gland but also involved in local cellular processes in the gland determining its size, morphogenesis or function.

Indeed, that possibility is substantiated by reports stressing the importance of TGFB signaling (including Activin, TGFs and BMPs) for development and function of the thyroid (Nicolussi A *et al.*, 2003; Mincione G *et al.*, 2003; Suzuki J *et al.*, 2005; Ma R *et al.*, 2009). These pathways have been recently shown to be modulated by IGSF1, at least in pituitary cell types like thyrotropes and gonadotropes (García M *et al.*, 2017A).

It is well established that TGFB1 is locally produced in the thyroid follicular cell in a paracrine fashion, where it regulates transcription over key genes of thyroid-specific differentiation and cell proliferation. TGFB1 signaling was shown to repress transcription the *TSHR* promoter but also of the *NIS* (sodium-iodide symporter) promoter (Suzuki J *et al.*, 2005; Nicolussi A *et al.*, 2003). Furthermore, TGFB1 negatively regulates the phosphorylation cascade and activation

of the IGF1/IGF1R signaling pathway in the thyroid, which also stimulates expression of the TSH receptor (Mincione G *et al.*, 2003).

Therefore, TGFB1 controls expression and signaling of three genes with essential roles in developmental growth and function of the thyroid gland (Postiglione MP *et al.*, 2002; Ravera S *et al.*, 2017). In fact, biallelic human mutations in *TSHR* lead to thyroid agenesis or hypoplasia of the gland associated with hyperthyrotropinemia (Krude H *et al.*, 1996; Biebermann H *et al.*, 1997), like present in our patients. Furthermore, the uptake isotopic-Iodide and -Technetium by the thyroid is also mediated by the NIS, localized at the apical membrane of follicular cells.

We recently showed that IGSF1 represses the negative effects of TGFB-Smad signaling on transcription of TRHR in pituitary thyrotropes, thereby allowing its physiological expression (García M *et al.*, 2017A). If IGSF1 in the thyroid follicular cell reproduces the same repressive effects over TGFB1-derived signaling, it is then plausible that IGSF1 also allows the physiological expression of TSHR in the thyroid (Figure 4). The likely effects of IGSF1 over factors and pathways involved in thyroid function and hormonosynthesis is consistent with IGSF1 preferential localization in the active follicles of the thyroid, where TSHR, NIS and IGF1R are abundantly express and where its actions would be necessary.

If the hypothesis is correct, then, mutations/deletions of IGSF1 in the thyroid would intrinsically lead to low expression of TSHR and hypoplasia and malfunction of the gland (low capacity for iodide uptake and synthesis of thyroid hormone).

Therefore, our finding that IGSF1 is expressed in the human thyroid opens the possibility to consider hypothyroidism caused by IGSF1 defects a especial type of “mixed” hypothyroidism with central (pituitary) and primary (thyroidal) components, whose characteristic features need to be investigated in detail for a comprehensive explanation of factors influencing the variable severity of each of the components and the cues governing their temporal clinical expression through life time.

In conclusion, from now onwards, investigations of thyroid hypoplasia within the IGSF1 syndrome need to take into account the expression of the protein in the thyroid gland in the present work, and that local effects of aberrant or absent IGSF1 in the thyroid tissue may (at least in part) be responsible for the reduced growth of the thyroid gland in IGSF1-deficient patients.

| P | Age | 15d | 1mo | 2mo | 6mo | 1y | *3y | 4y | *5y | 10y | 12y | 14y |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | **TSH** *mIU/L* (1.5-5.7) | - | 2.7 | **0** | **0.1** | **0.1** | **48** | **0.15** | **140** | **0.15** | **0.15** | **0** |
| | **T4** *µg/dl* (4.5-14) | - | **3.3** | 7 | 6 | 8.9 | **2.3** | 9 | **1.49** | 8.9 | 6.3 | 5.6 |
| | **FT4** *pmol/L* (11-25) | - | **4** | - | 14 | - | **0.46** | - | **1.91** | 15.2 | 16.3 | 14.9 |
| | **FT3** *pmol/L* (2.6-8) | - | **0.64** | - | 7.6 | - | **1.1** | - | **1.1** | - | - | - |
| | **L-T4 dose** µg/Kg/day | - | 0 | 6.25 | 3.8 | 3.4 | 0 | 2.6 | 0 | 2.7 | 2.4 | 2.0 |
| | **Testis vol.** *ml* (p98 upon age) | - (2.8) | - (2.8) | - (2.8) | - (2.8) | 2/**3** (2.8) | **4/4** (2.8) | - (3) | - (3.2) | **5/5** (5) | 12/**15** (15) | 20/20 (28) |
| 2 | **TSH** *mIU/L* (1.5-5.7) | 2.8 | 5.4 | **0** | **0.15** | **0.15** | **81.8** | **0.1** | **>150** | **0.1** | **0** | **<0.5** |
| | **T4** *µg/dl* (4.5-14) | **3.4** | **3.3** | 8.1 | 6.9 | 9.3 | **<1.0** | 7 | **0.1** | 7 | 8.2 | 9 |
| | **FT4** *pmol/L* (11-25) | **0.4** | - | - | 15.6 | - | **1.03** | - | **<1.0** | 12.2 | 18.3 | 13.1 |
| | **FT3** *pmol/L* (2.6-8) | **2.4** | - | - | 7.8 | - | **0.8** | - | **0.6** | - | - | - |
| | **L-T4 dose** µg/Kg/day | 0 | 4.01 | 4.06 | 3.9 | 3.3 | 0 | 2.5 | 0 | 2.4 | 2.5 | 2.2 |
| | **Testis vol.** *ml* (p98 upon age) | - (2.8) | - (2.8) | - (2.8) | - (2.8) | **3/3** (2.8) | - (2.8) | - (3) | - (3.2) | **5/5** (5) | **15/15** (15) | 20/20 (28) |

**Table 1: Long-term follow up of thyroid axis profiles of patients with IGSF1 defect.** Hormone parameters are represented in chronological order along patient's life. In blue and red are represented hormone values below and above normal ranges, respectively. * Age of re-evaluation without treatment. TSH: thyrotropin, T4: total thyroxine, FT4: free thyroxine, FT3: free triiodothyronine, L-T4 dose: dose of levothyroxine treatment. Testis vol.: testicular volume. Reference range for testicular volume following Joustra SD *et al.*, 2015.

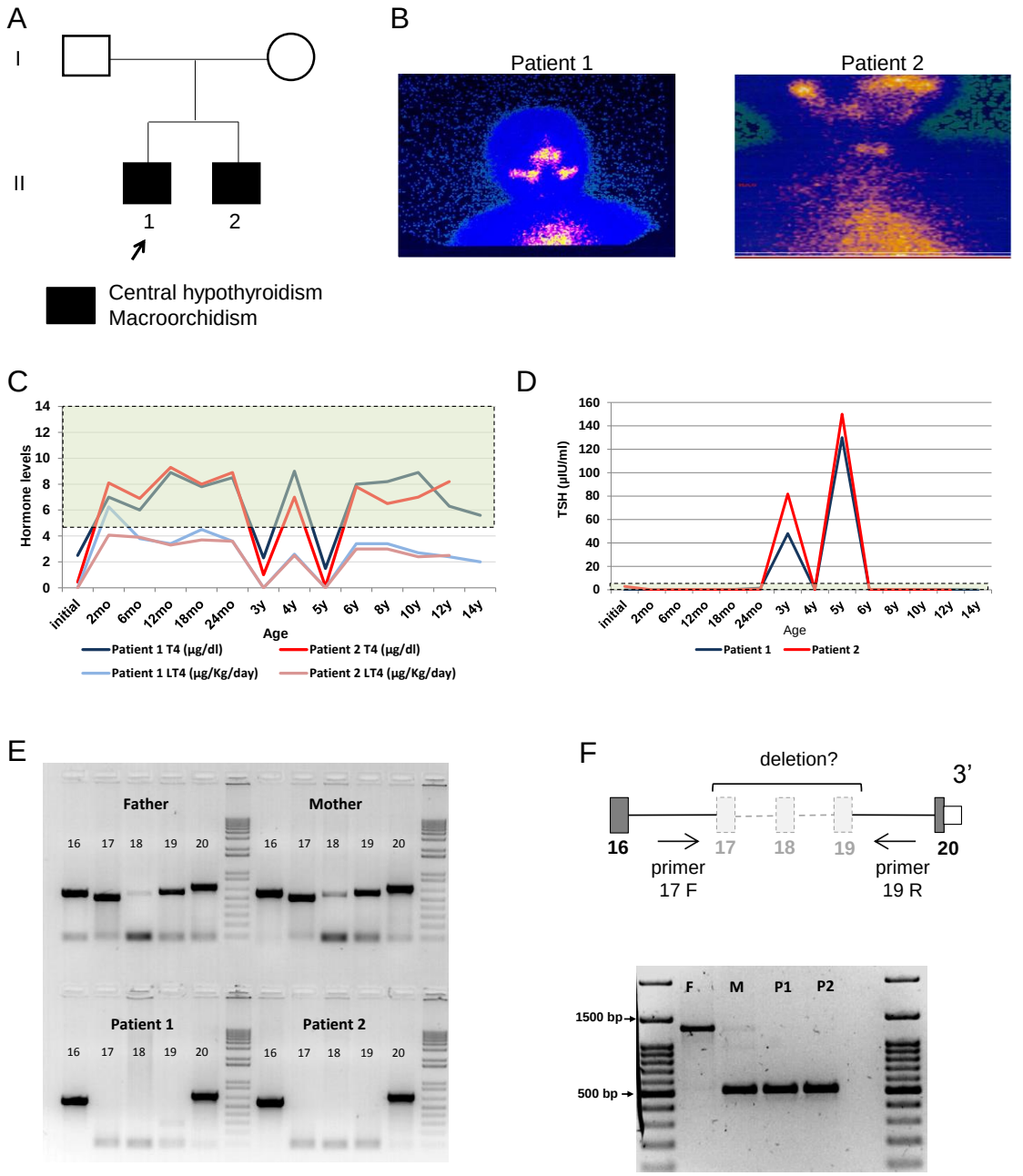



**Figure 1: Clinical and genetic characterization of two patients with IGSF1 X-linked syndrome.** (A) Non-consanguineous familiar pedigree of patients showing two affected brothers with central congenital hypothyroidism and macroorchidism. (B) Thyroid scintigraphic imaging with 99-Tc in the two brothers, showing thyroid hypoplasia in patient 1 and apparent agenesis of the gland in patient 2. (C) Follow-up of T4 values through time (dark blue and red colors in patient 1 and 2, respectively), which vary according to LT4-treatment dose (light blue and red colors in patient 1 and 2, respectively), showing severe hypothyroidism when the treatment was withdrawn in both patients. (D) Follow-up of TSH values through time, showing a suppression of TSH during L-T4 treatment and a remarkably hyperthyrotropinemia when the treatment was withdrawn in both patients. Green area represents normal range for total T4 values (C) and TSH values (D), (E) PCR of *IGSF1* gene visualized by agarose gel electrophoresis, showing the absence of amplification of the exons 17, 18 and 19 in patient 1 and patient 2, compared with the positive amplification in both parents. (F) Confirmation of the IGSF1 deletion in patients and their mother by PCR using specific primers flanking the exons 17 and 19. DNAs of both patients and their mother were carrier of the deletion amplifying in a short fragment (600 bp), whereas the DNA of the father was unaffected amplifying in a long fragment containing all exons (1400 bp).

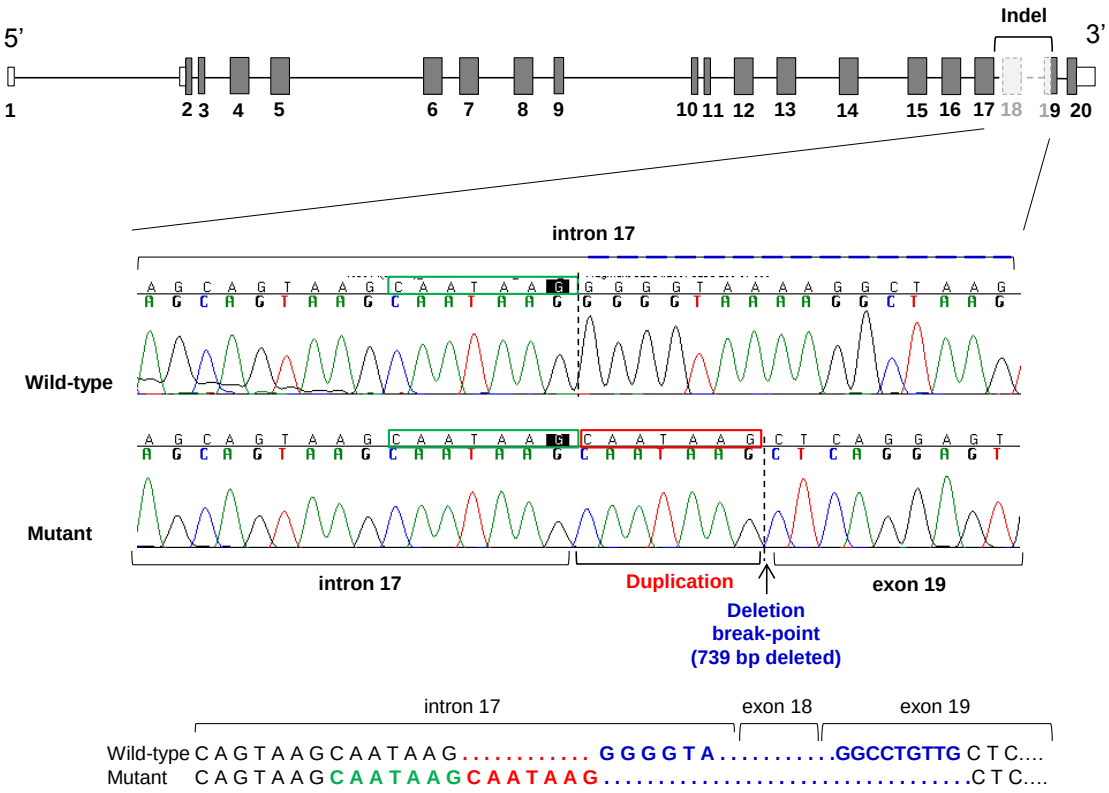



**Figure 2: Break-point and structure of IGSF1 INDEL.** The INDEL defect identified is located between exons 17 and 19 near to 3'-UTR of the gene. Sanger sequencing showed in both patients a hemizygous duplication of seven base pairs (CAATAAG) in intron 17 (red square and letters), followed by a deletion of 739 bp (dotted blue line) including the complete sequence of exon 18 and 9 bp of exon 19 (blue letters) of *IGSF1* gene.

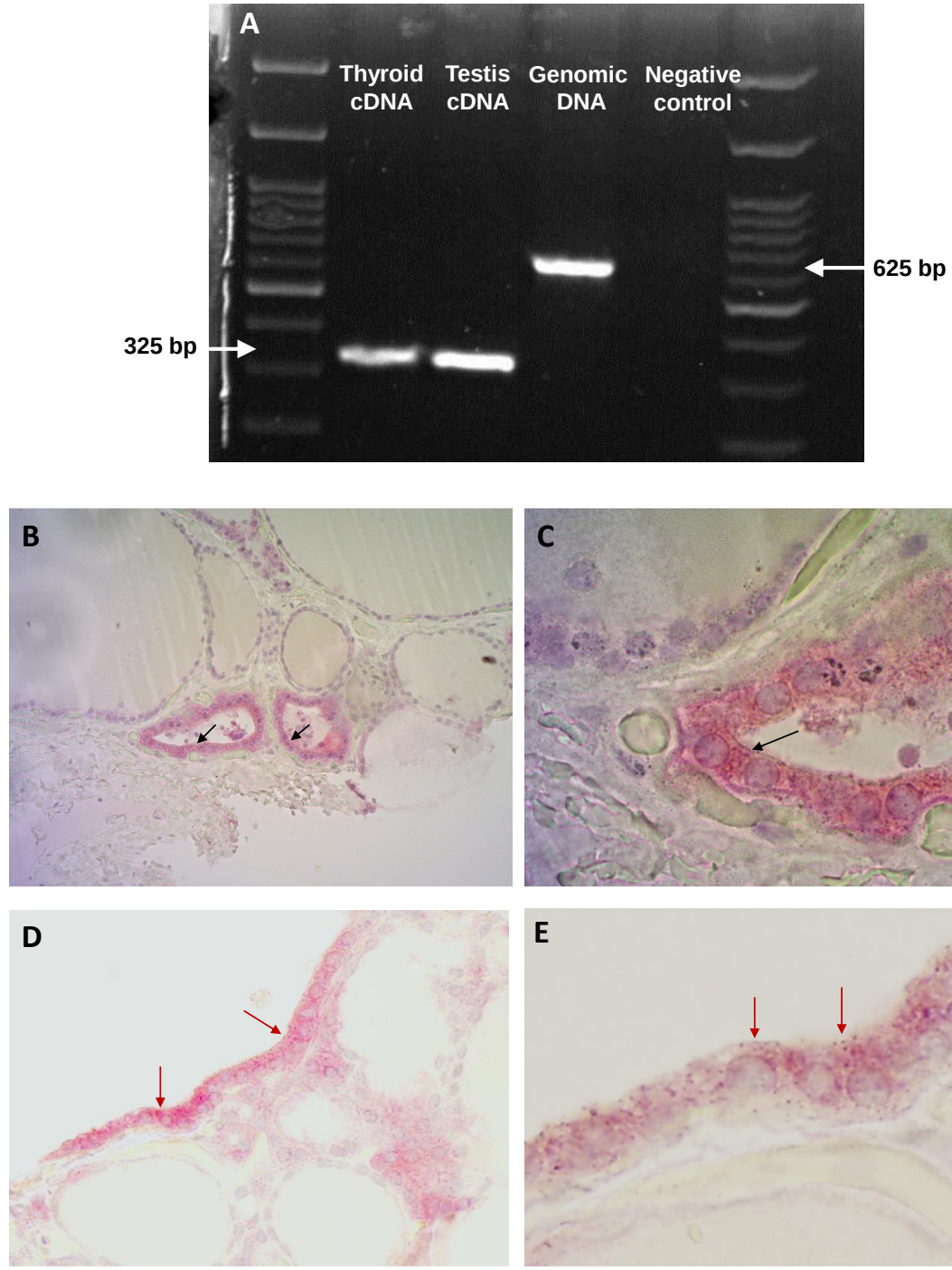



**Figure 3: IGSF1 is expressed in the thyroid gland.** (A) Agarose gel electrophoresis showing a PCR product obtained from gDNA amplification with specific exonic primers for IGSF1 (*primer forward* for exon 2 and *primer reverse* for exon 3), reveling that *IGSF1* cDNA is present in human thyroid, as it is in human testis (control). A fragment of 325 bp corresponding with mRNA after splicing of intron 2-3 was amplified from cDNA. Normal genomic DNA was used as control for genomic DNA contamination in the thyroid and testis cDNA templates, amplifying the expected larger fragment of 625 bp, corresponding with part of exons 2 and 3 connected by intron 2-3. (B, C, D, E) Immunohistochemistry of human thyroid gland showing IGSF1 expression in the thyrocytes of the active follicles, with higher epithelium (black arrows in B and C panels). The expression of the protein is cytoplasmic, showing a granular pattern with some apical reinforcement (red arrows in D and E panels). IGSF1 positivity is showed in pink and hematoxylin counterstained nuclei in purple.

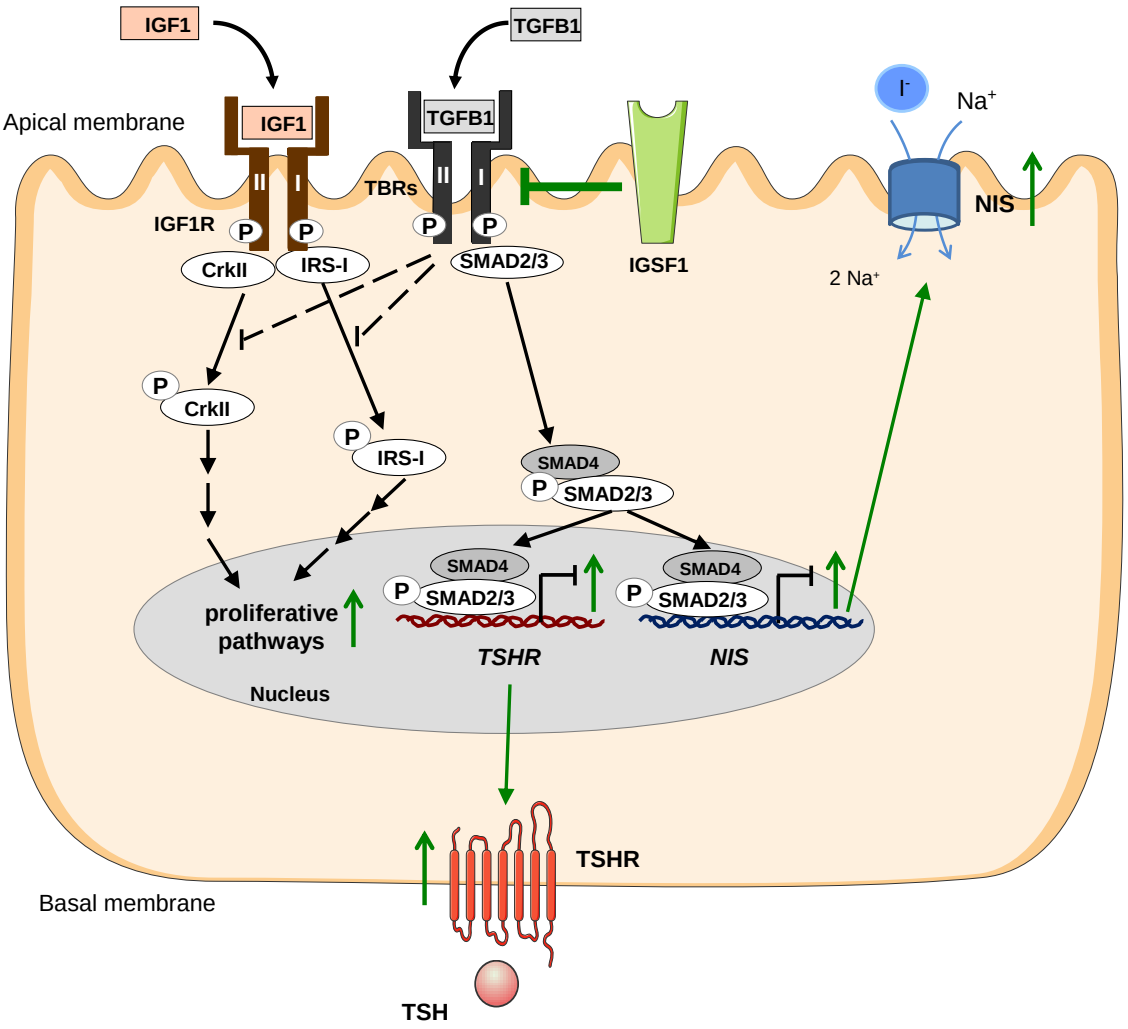
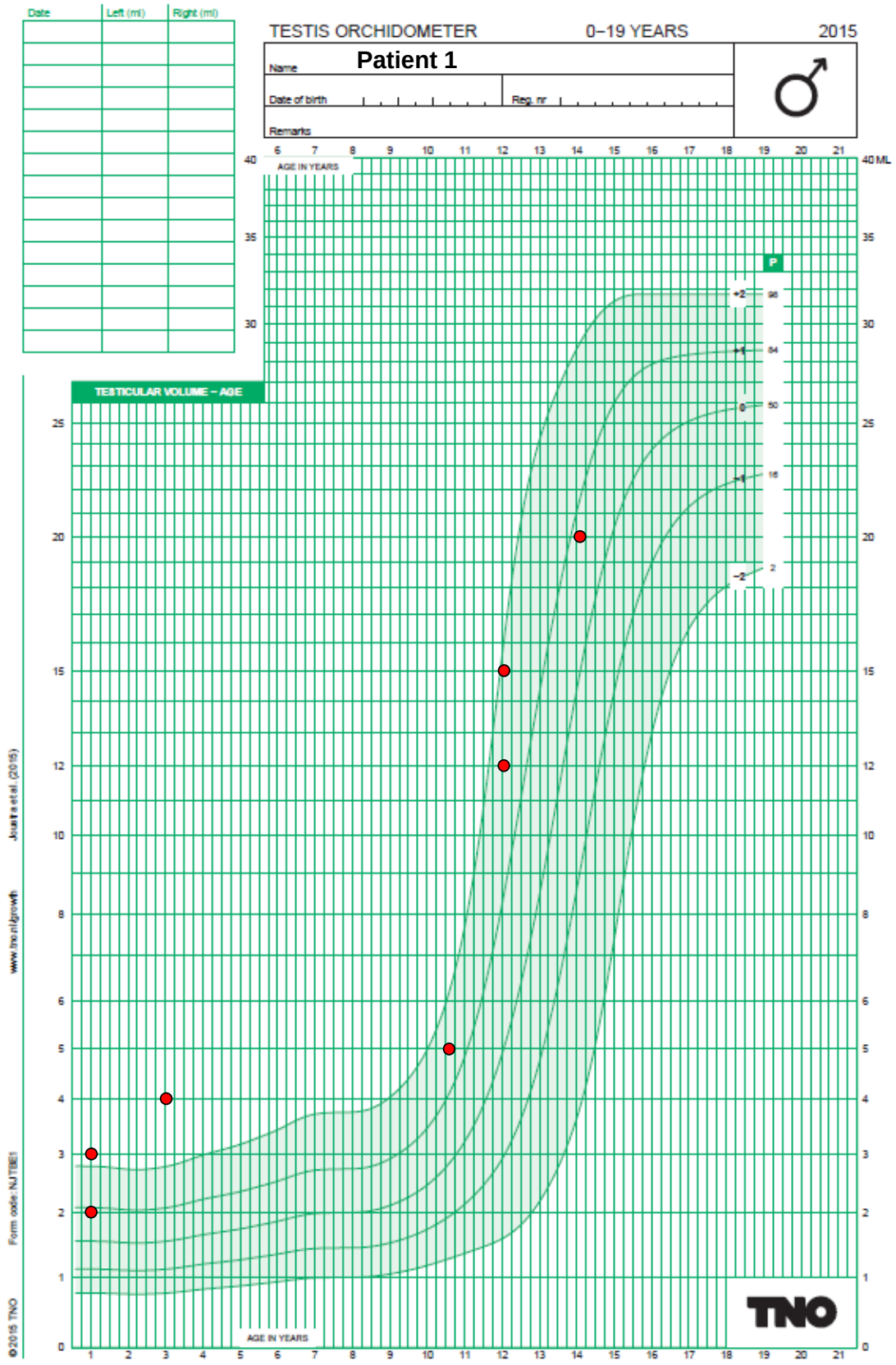
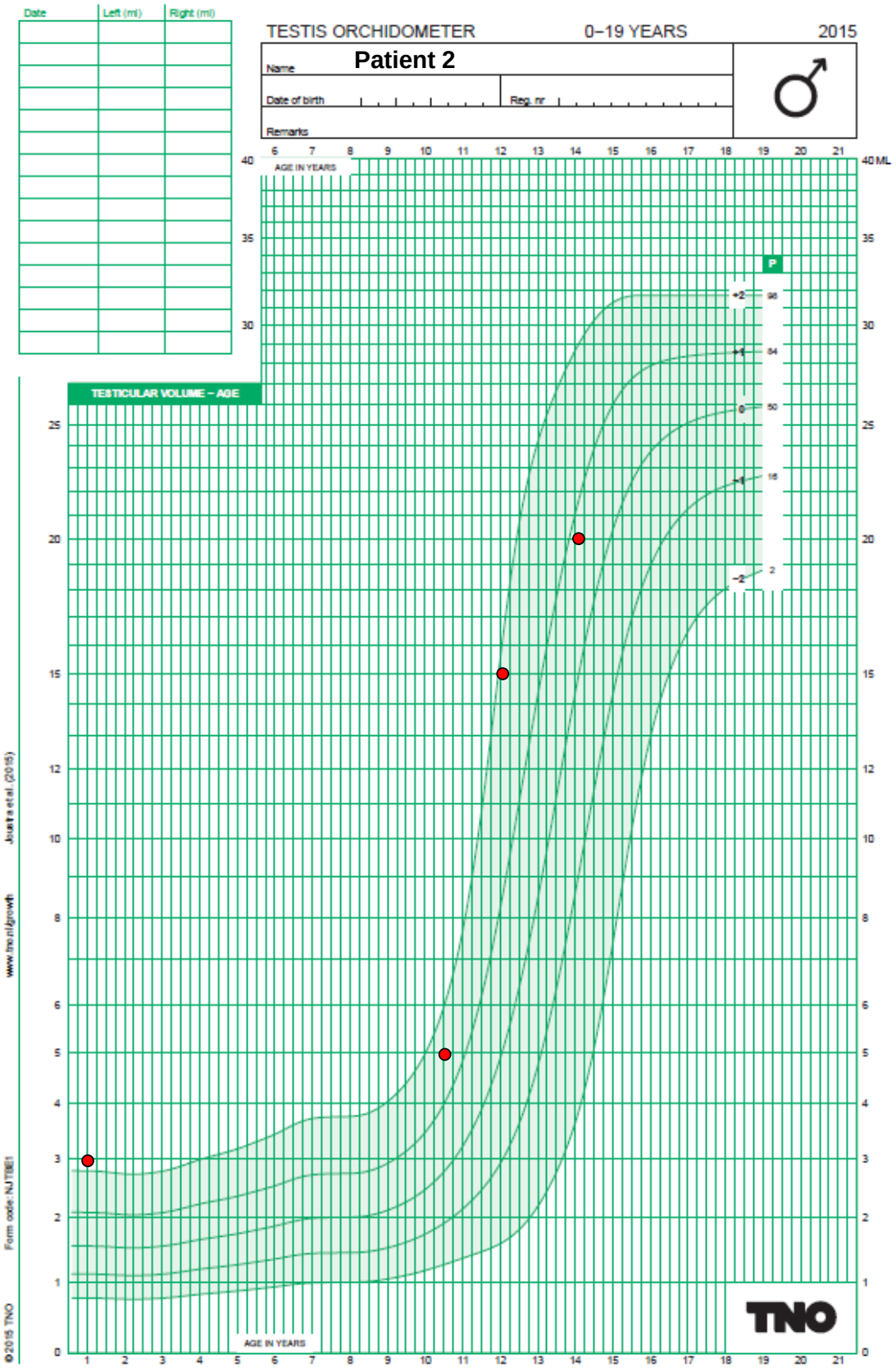


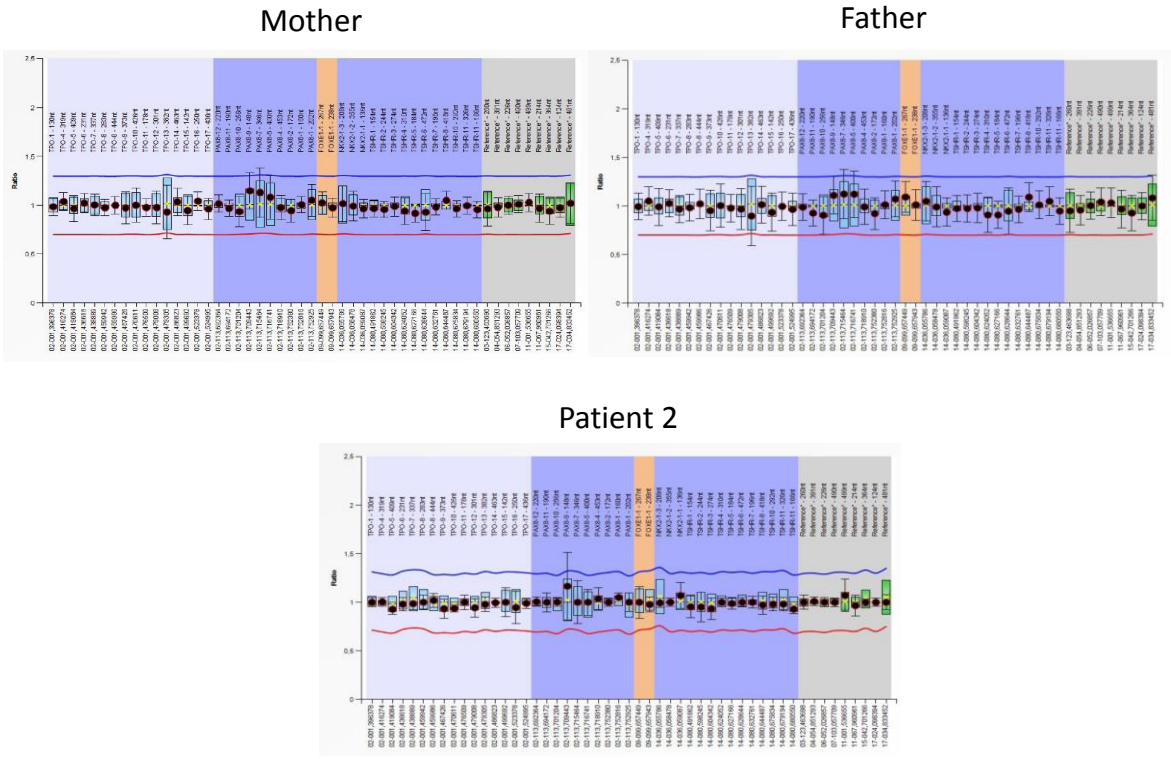

**Figure 4: Hypothesized model for IGSF1 functions and molecular mechanisms in thyrocites of the thyroid gland.** In thyrocites, TGFB1 activates TGFB receptors (TBRs) and stimulates Smad2/3 signaling cascade leading to repression of the *TSHR* gene, which reduces the TSH-TSHR signaling, and *NIS* gene, which reduces iodine uptake. Moreover, TGFB1-TGFBR interacts with IGF1-IGF1R signaling pathway reducing tyrosine phosphorylation of IGF1R substrates (including IRS-1 and CrkII), which leads to negative regulation of cell proliferation. IGSF1 (green symbols) would repress TGFB1 pathway, positively regulating *TSHR* and *NIS* expression and therefore enhancing TSH-TSHR signaling and iodine uptake, which conduce to thyroid hormone synthesis and cellular differentiation and proliferation. Moreover, repression of TGFB1-TGFBR pathway by IGSF1 leads to increase phosphorylation of IGF1R substrates, enhancing cellular proliferation.




**Supplemental Figure 1: Long-term follow up of patient 1's testicular size.** Patient with *IGSF1* defect developed a progressive macroorchidism observed since 1 years of age, first unilateral with 3 ml (>p98) of one testicle size, which evolved bilateral and continued increasing near the percentile 84 until 14 years of age. Reference chart for testicular volume from Joustra SD *et al.* 2015.




**Supplemental Figure 2: Long-term follow up of patient 2's testicular size.** Patient with *IGSF1* defect developed a progressive macroorchidism observed since 1 years of age, with a testicular size of 3 ml (>p98), which continued increasing near the percentile 84 until 14 years of age. Reference chart for testicular volume from Joustra SD *et al.* 2015.




**Supplemental Figure 3: MLPA from parents and patient 2 ruling out defects in genes related to thyroid dysgenesis (TPO, PAX8, FOXE1, NKX2.1 and TSHR).**

# CAPÍTULO IV

## Hipotiroidismo central debido a una mutación en TRHR que altera la afinidad del receptor por su ligando y la transactivación de la Gq

El hipotiroidismo congénito central (HCC) es un trastorno infradiagnosticado caracterizado por una producción deficiente de TSH que conduce a una reducción en la síntesis de hormonas tiroideas. Los defectos en el receptor de TRH (TRHR) son muy infrecuentes y se heredan de forma recesiva, generalmente se asocian con HCC identificado de forma accidental por talla baja en la infancia.

En este estudio hemos caracterizado clínica y genéticamente a una familia consanguínea de etnia gitana con hipotiroidismo central. Además, hemos identificado los mecanismos moleculares subyacentes a la enfermedad, determinando la patogenicidad de la mutación identificada. El caso índice es un niño de 8 años de edad en el que se identificó una mutación en homocigosis en el gen TRHR que produce un cambio de aminoácido (c.392T>C, p.I131T). El caso índice presentaba hipotiroidismo central moderado (TSH: 2,61 mIU/L, Normal: 0,27-4,2; FT4: 9,52 pmol/L, Normal: 10,9-25,7) y sobrepeso (IMC: 20,4 kg/m2, p91), pero estatura normal (122 cm; -0,58 DS).Su madre, dos hermanos y su abuela paterna fueron heterocigotos para la mutación y presentaron hipertirotropemina aislada (TSH: 4.3-8 mUI/L). La mutación I131T, en el bucle intracelular 2 de TRHR, disminuye la afinidad de TRH e incrementa la mitad de la concentración eficaz máxima (EC50) para la señalización. El modelo de los complejos TRHR-Gq predice que la mutación interrumpe la interacción entre el receptor y el bolsillo hidrofóbico formado por la Gq.

En conclusión, hemos identificado en una familia consanguínea un nuevo defecto de cambio de aminoácido en el TRHR asociado a hipotiroidismo central en homocigotos y a hipertirotropinemia en heterocigotos, lo que sugiere una elevación compensatoria de TSH pero con una biopotencia reducida. La mutación I131T disminuye la unión del ligando TRH y el acoplamiento y señalización de TRHR-Gq.

*García M, González de Buitrago J, Jiménez-Rosés M, Pardo L, Hinkle PM, Moreno JC. Central hypothyroidism due to TRHR mutation causing impaired ligand affinity and transactivation of Gq. J Clin Endocrinol Metab. 2017. (Epub ahead of print).*

# Central Hypothyroidism Due to a TRHR Mutation Causing Impaired Ligand Affinity and Transactivation of Gq

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**Context:** Central congenital hypothyroidism (CCH) is an underdiagnosed disorder characterized by deficient production and bioactivity of thyroid-stimulating hormone (TSH) leading to low thyroid hormone synthesis. Thyrotropin-releasing hormone (TRH) receptor (*TRHR*) defects are rare recessive disorders usually associated with incidentally identified CCH and short stature in childhood.

**Objectives:** Clinical and genetic characterization of a consanguineous family of Roma origin with central hypothyroidism and identification of underlying molecular mechanisms.

**Design:** All family members were phenotyped with thyroid hormone profiles, pituitary magnetic resonance imaging, TRH tests, and dynamic tests for other pituitary hormones. Candidate *TRH*, *TRHR*, *TSHB*, and *IGSF1* genes were screened for mutations. A mutant TRHR was characterized *in vitro* and by molecular modeling.

**Results:** A homozygous missense mutation in *TRHR* (c.392T > C; p.I131T) was identified in an 8-year-old boy with moderate hypothyroidism (TSH: 2.61 mIU/L, Normal: 0.27 to 4.2; free thyroxine: 9.52 pmol/L, Normal: 10.9 to 25.7) who was overweight (body mass index: 20.4 kg/m$^2$, p91) but had normal stature (122 cm; –0.58 standard deviation). His mother, two brothers, and grandmother were heterozygous for the mutation with isolated hyperthyrotropinemia (TSH: 4.3 to 8 mIU/L). The I131T mutation, in TRHR intracellular loop 2, decreases TRH affinity and increases the half-maximal effective concentration for signaling. Modeling of TRHR-Gq complexes predicts that the mutation disrupts the interaction between receptor and a hydrophobic pocket formed by Gq.

**Conclusions:** A unique missense *TRHR* defect identified in a consanguineous family is associated with central hypothyroidism in homozygotes and hyperthyrotropinemia in heterozygotes, suggesting compensatory elevation of TSH with reduced biopotency. The I131T mutation decreases TRH binding and TRHR-Gq coupling and signaling. **(*J Clin Endocrinol Metab* 102: 1–10, 2017)**

Central congenital hypothyroidism (CCH) is caused by deficient production of thyroid hormones (T4 and T3) due to low synthesis, secretion, or bioactivity of thyrotropin (thyroid-stimulating hormone [TSH]). CCH is an underdiagnosed disorder because CCH patients are not detected by TSH-based neonatal screening programs for congenital hypothyroidism (CH) implemented in most countries (1). However, uncommon T4-based CH screening programs in few countries recently estimated the prevalence of CCH as 1 in 16,000 to 30,000 newborns (2, 3). The

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Abbreviations: CCH, central congenital hypothyroidism; CH, congenital hypothyroidism; FT4, free thyroxine; GPCR, G-protein–coupled receptor; ICL, intracellular loop; IP3, phosphatidylinositol; L-T4, levo-thyroxine; LH, luteinizing hormone; MD, molecular dynamics; PDB ID, Protein Data Bank Identification; PKC, protein kinase C; PRL, prolactin; TMD, transmembrane domain; TRH, thyrotropin-releasing hormone; TRHR, thyrotropin-releasing hormone receptor; TSH, thyroid-stimulating hormone.

paucity of clinical cases identified and the complexity of hypothalamic-pituitary regulation of the thyroid axis leaves the molecular mechanisms underlying CCH largely unknown (4). At present, genetic defects in only four genes have been identified in patients with isolated CCH: *TSHB* (encoding the B-subunit of the TSH glycoprotein hormone), *TRHR* (the specific 7-transmembrane domain receptor for hypothalamic thyrotropin-releasing hormone [TRH]), *IGSF1* (a protein regulating the expression of TRHR in pituitary thyrotropes), and the recently identified *TBL1X* (a subunit of the NCoR-SMRT complex) (5). Most patients described with CCH harbored defects in *TSHB* and *IGSF1*, but three families with recessively inherited *TRHR* defects have been identified (6–8).

The TRH receptor (TRHR) is a G-protein–coupled receptor (GPCR) located at pituitary thyrotropes and activated by hypothalamic TRH. TRHR contains an extracellular N terminus, seven transmembrane domains (TMDs), three extracellular loops, three intracellular loops (ICLs), and a cytoplasmic tail. TRH interacts with amino acids of the extracellular loops and then moves into the TMD binding pocket (9, 10) triggering small, local structural changes near the binding site that are translated into larger-scale helix movements at the intracellular site, mainly TMDs 5 and 6, opening a cavity for the binding of the C-terminal $\alpha 5$ helix of the G-protein (11). The formation of the TRH-TRHR-Gq complex triggers activation of the phosphatidylinositol (IP3)-calcium-protein kinase C (PKC) pathway (12).

TRH-TRHR signaling promotes the synthesis, secretion, and bioactivity of TSH, all necessary for the proper synthesis of T4 and T3 in the thyroid gland (13, 14). The three unrelated patients with identified TRHR defects were missed in TSH-based neonatal screening programs (6–8). The first two patients described were referred to clinicians at the ages of 9 and 11 years with short stature and variable symptoms, consistent with hypothyroidism (lethargy, fatigue, poor school performance), whereas the third was diagnosed and treated for CH at 2 months of life (8). In all cases, thyroid hormone profiles revealed normal TSH (with suspected low bioactivity) and the presence of moderate hypothyroidism. Heterozygous carriers were reportedly euthyroid.

The first patient described was a compound heterozygote for an early stop codon in the *TRHR* (p.R17X) and an inframe deletion added to a missense change (p.S115-T117del + p.A118T) in the other allele (6). The same p.R17X mutation was found in the second patient in homozygous state (7), whereas the third had a homozygous missense mutation (p.P81R) (8). All TRHR mutations identified so far severely impaired TRHR signaling (6–8).

Here we present a unique missense mutation in TRHR located at a highly conserved hydrophobic position (Φ) at the (E/D)$R^{3.50}YX_5P\Phi XY$ motif of GPCRs, which reduces affinity for TRH and impairs, but not fully abrogates, signal transduction by the receptor. Consistent with this residual function, the mutation causes moderate CH in the homozygous state and central hyperthyrotropinemia in heterozygotes, suggesting compensatory elevation of TSH with reduced biopotency.

## Materials and Methods

### Informed consent

Informed consent for genetic studies was obtained from the index patient and his family, according to protocols followed at the San Pedro de Alcántara Hospital (Cáceres, Spain), where the patient was clinically followed.

### Hormonal determinations and TRH test

TSH, free thyroxine (FT4), luteinizing hormone (LH), follicle-stimulating hormone, and prolactin (PRL) were determined in serum by electrochemiluminescence with the Elecsys-170 platform (Roche, Basel, Switzerland). IGF-1, IGFBP-3, adrenocorticotropic hormone, and cortisol were measured by chemiluminescence with the Immulite 2000 system (Siemens, Munich, Germany). The TRH stimulation test was performed as previously reported (15). An indirect measure of TSH bioactivity was calculated through the percentage increase of serum FT4 180 minutes after TRH administration, as reported (16).

### Mutation screening

All coding regions of *TRH* (the gene encoding the TRH), *TRHR* (encoding the TRH receptor), *TSHB* (encoding the specific TSH $\beta$ subunit), and *IGSF1* (coding for the immunoglobulin superfamily factor 1) were amplified by PCR using appropriate primers flanking each exon. PCR products were purified and directly sequenced on an automated DNA sequencer (3100 Genetic Analyzer; Applied Biosystems).

DNA samples of the index patient and his mother were used for next-generation sequencing (NextSeq-500). A panel of 320 thyroidal genes, of our own design, including *TG*, *TPO*, *NIS*, *DUOX2*, *DUOXA2*, *DEHAL1*, *TSHR*, and *GNAS*, was used.

### Evaluation of TRHRs

*In vitro* activity of wild-type and mutant hemagglutinin-tagged TRHRs was evaluated using fixed-cell enzyme-linked immunosorbent assay to measure plasma membrane expression, [$^3$H]Me-TRH binding to measure agonist affinity, and an AP1-luciferase reporter assay to measure signal transduction, as described previously (17–19) and in the Supplemental Methods.

### Computational model of wild-type and I131T mutant TRH receptors in complex with TRH and Gq

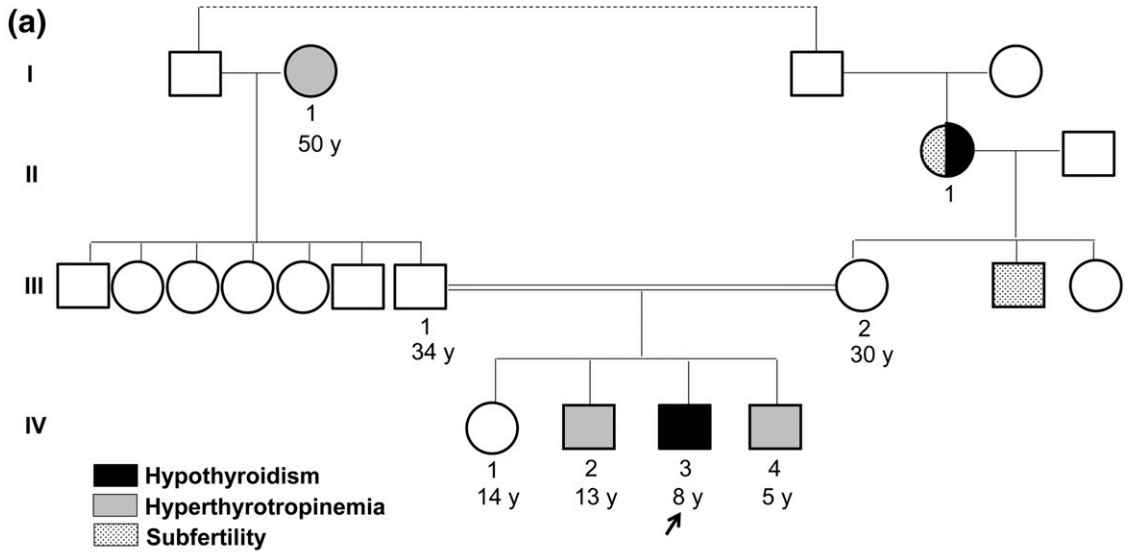
The "active-like" state of human TRHR (UniProt entry P34981) in complex with TRH and Gq was built using a combination of structural templates. Crystal structures of active $\mu$-opioid receptor [Protein Data Bank Identification (PDB ID): 5C1M] (20), the complex between $\beta_2$-adrenergic receptor and Gs protein (PDB ID: 3SN6) (11) and Gq protein (PDB ID: 3AH8) (21), were used (Supplemental Methods). TRH was docked into the "active-like" conformation of TRHR using MOE (Chemical Computing Group Inc., Montreal, QC,

Canada) (Supplemental Methods). To evaluate the effect of the I131$^{ICL2}$T mutation in the TRHR-Gq interface, we performed molecular dynamics (MD) simulations of wild-type and mutant receptors (Supplemental Methods).

## Results

### Clinical case

The index case is a male of Roma descent, the third sibling of a consanguineous kindred [Fig. 1(a)]. He was not detected by a TSH-based neonatal screening program (using TSH threshold >7 mIU/L). At the age of 8 years, he was referred to the pediatrician for evaluation of abnormal thyroid function. Hormonal tests revealed mild hypothyroidism (FT4: 9.52 pmol/L [Normal: 10.9 to 25.7 pmol/L] and TSH: 2.61 mIU/L [Normal: 0.27 to 4.2 mIU/L]) [Fig. 2(a)] in the absence of antithyroid antibodies. He was overweight (body mass index: 20.4 kg/m$^2$, p91) but had normal stature (122 cm, –0.58 standard deviation)




| Case | I.1 | III.1 | IV.1 | IV.2 | IV.3 | IV.4 | III.2 |
|---|---|---|---|---|---|---|---|
| **TSH** mU/L (0.27-4.2) | 5.17 | 1.3 | 2.95 | 8.05 | 2.61 | 4.65 | 2.71 |
| **FT4** pmol/L (10.9-25.7) | N | 13.88 | 17.73 | 13.62 | 9.52 | 12.46 | 14.03 |
| **Height** cm (-2…+2 SD) | - | 171 -0.76 | - | - | 122 -0.58 | - | 155.4 -1.02 |
| **Genotype p.I131T (c.392T>C)** | +/- | +/- | +/- | +/- | +/+ | +/+ | +/- |

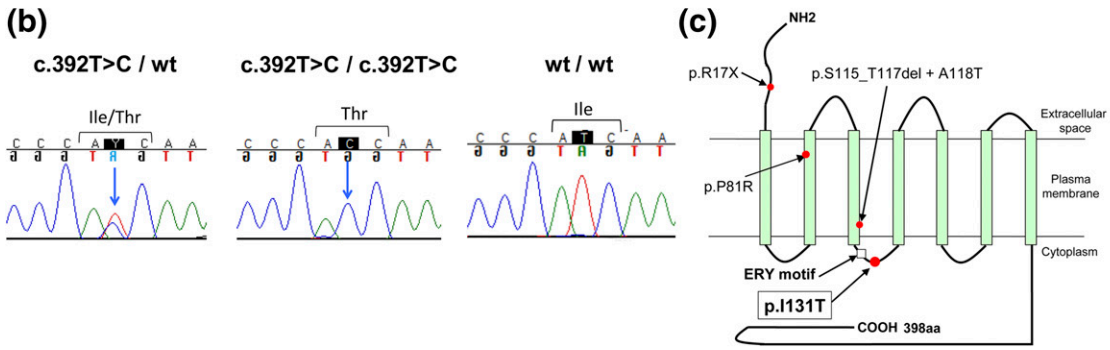



**Figure 1.** Clinical and biochemical features of a family with TRHR defect. (a) Phenotype of members of the pedigree showing consanguinity, expressed as a double line linking symbols for mother and father of the index patient (arrow). Thyroid hormone profile of several members of the family. Blue and red represent hormone values below and above normal ranges, respectively. (b) Representative chromatograms showing the wild type and the I131T TRHR mutation in heterozygous and homozygous state. (c) Scheme showing the location of the I131T mutation at the second ICL of the TRHR (highlighted) and other TRHR mutations previously described. Abbreviations: $^{+/-}$, heterozygous mutation; $^{+/+}$, homozygous mutation; wt, wild type; y, year.

(a)

| N | Age (years) | TSH mU/L | FT4 pmol/L |
|---|---|---|---|
| N | - | 0.27-4.2 | 10.9-25.7 |
| 1 | 6.2 | 4.49 | 10.68 |
| 2 | 7.4 | 2.35 | 9.91 |
| 3 | 7.7 | 2.67 | 9.27 |
| 4 | 7.7 | 3.40 | 9.27 |
| 5 | 8 | 2.61 | 9.52 |
| 6 | 10 | 5.68 | 10.55 |

(b)

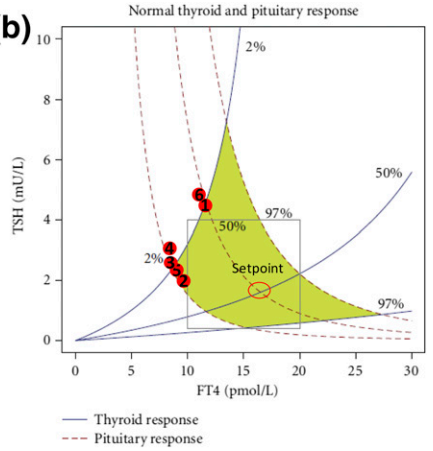



**Figure 2.** Historical thyroid hormone profiles of index patient from 6 to 10 years of age. (a) TSH and FT4 values during 4 years follow-up. All values were determined when the patient was not treated with L-T4. (b) Graphical correlation between TSH and FT4 values plotted in chart for normal thyroid and pituitary responses (adapted from 22). Blue and red represent hormonal values below and above normal ranges, respectively.

[Fig. 1(a)]. The patient did not present symptoms of hypothyroidism; however, all available TSH-FT4 paired determinations fell outside the area representing the normal dynamic relation between the two parameters, following the model of Dietrich *et al.* (22) [Fig. 2(b)]. The patient displayed normal TSH response to TRH (Supplemental Fig. 1). The adrenocorticotropic hormone dynamic test and basal PRL, follicle-stimulating hormone, LH, IGF-1, and IGFBP-3 determinations were normal. His testicular volume was 3 mL, consistent with a prepubertal stage. Brain magnetic resonance imaging showed normal size and shape of the pituitary and thyroid ultrasounds revealed normal thyroid size and structure (data not shown). He was started on levo-thyroxine (L-T4) replacement (50 μg/d), and 1 month later, his FT4 levels reached normal ranges (13.77 pmol/L) at the expense of suppressed TSH (0.07 mIU/L), a characteristic feature of treated central hypothyroidism (1). When the patient was 10 years of age, L-T4 treatment was withdrawn for 1 month, and his thyroid function was re-evaluated, showing elevated TSH levels and decreased FT4 but normal TSH and PRL responses to a second TRH test (Supplemental Fig. 2). As part of the test, FT4 was measured before (0 minutes) and 3 hours after (180 minutes) TRH administration, and the percentage FT4 increase was calculated as an indirect measure of TSH bioactivity, as reported (16). The FT4 increase was below the normal range (Supplemental Fig. 2).

Remarkably, four members of the family (two siblings, mother, and paternal grandmother) showed mild hyperthyrotropinemia with normal FT4 [Figs. 1(a) and 3]. Retrospectively analyzed, the patient also displayed hyperthyrotropinemia at 6 years of age [Fig. 2(a)].

All siblings of the patient were hormonally re-evaluated on two occasions. In the first one, only one out of four showed hyperthyrotropinemia (Supplemental Fig. 2), whereas 6 months later, two out of four showed elevated TSH, indicating the intermittent nature of hyperthyrotropinemia in these individuals (Supplemental Table 1). Thyroid autoimmunity was negative in all family members. No goiter was present in the siblings at ultrasounds, according to age- and sex-specific reference intervals (24, 25) (Supplemental Table 1). Four of the five siblings, including the index patient, are currently obese (as defined as body mass index centile >95). Interestingly, the only child with normal weight also presented hyperthyrotropinemia, suggesting that TSH elevation in this family is not directly associated with obesity (Supplemental Table 1).

TRH tests were also performed in siblings and parents, showing normal TSH and PRL responses to TRH (Supplemental Fig. 2). TSH bioactivity was evaluated from the TRH test (Supplemental Fig. 2). FT4 increases were below the normal ranges, again suggesting low TSH biopotency in all serum samples available (16).

Two relatives of this consanguineous pedigree were treated with L-T4: the paternal grandmother and the patient's mother during pregnancy with her fifth child when her TSH levels remained slightly elevated during treatment with 25-μg/d L-T4 (Fig. 3). No difficulties with lactation were reported. The maternal grandmother had hypothyroidism in her youth and difficulties becoming pregnant for more than 6 years after marriage. Thereafter, she experienced one spontaneous abortion and three fruitful pregnancies. One of the sons of the maternal grandmother has similar subfertility complaints [Fig. 1(a)].

## Identification of the TRHR mutation

Direct sequencing of the coding exons of four candidate genes for central hypothyroidism (*TRH*, *TRHR*, *TSHB*, and *IGSF1*) revealed a homozygous missense mutation in the *TRHR* gene of the patient (c.392T > C),

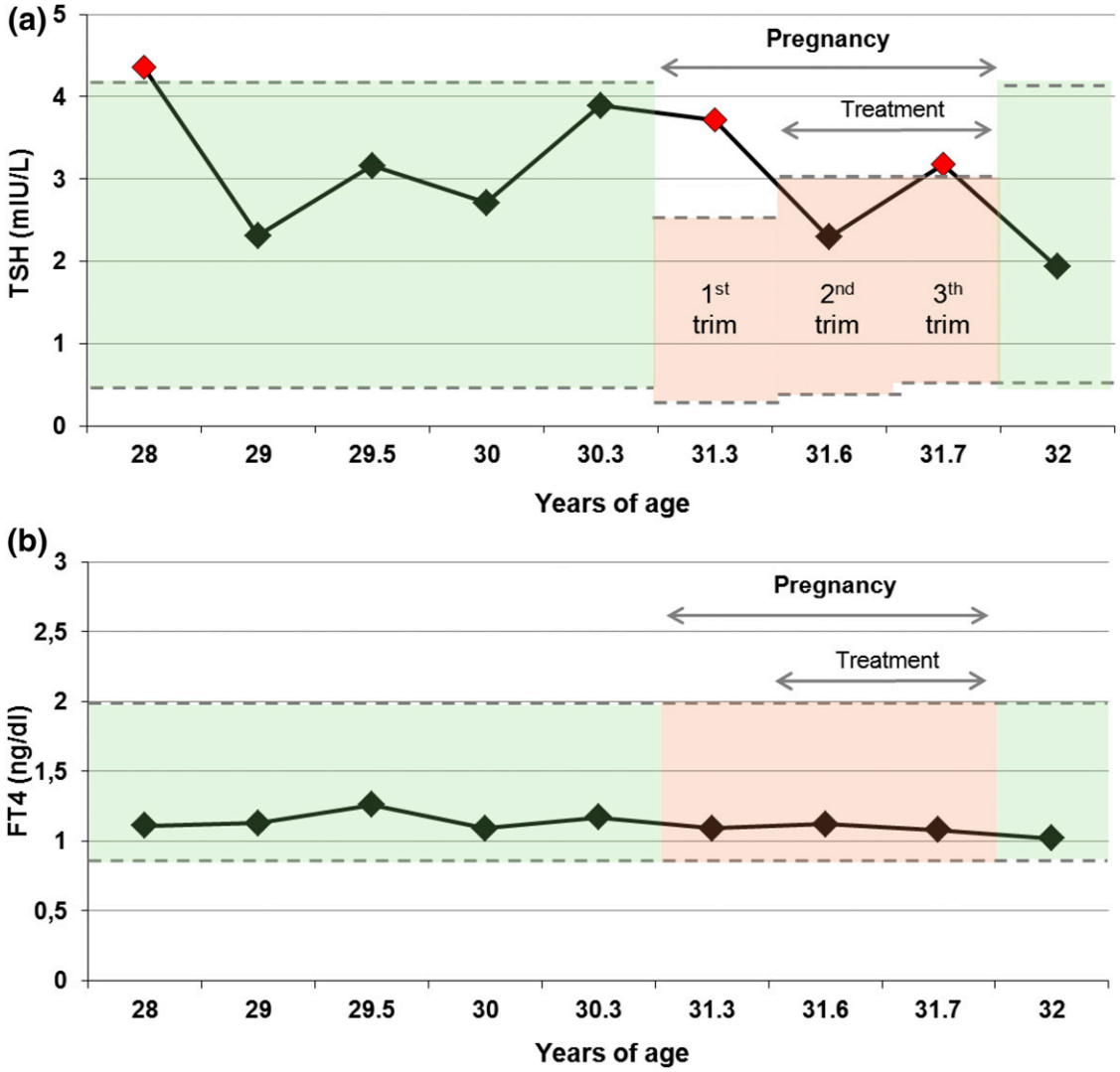



**Figure 3.** Longitudinal thyroid hormone profile of the patient's mother over 4 years, including pregnancy. (a) TSH follow-up profile. Green area represents the normal ranges for healthy adult individuals (0.27 to 4.2 mU/L). Red area represents the normal ranges for healthy pregnant women (first trimester: 0.1 to 2.5 mU/L, second trimester: 0.2 to 3 mU/L, third trimester: 0.3 to 3 mU/L, following American Thyroid association recommendations) (23). (b) Free T4 follow-up profile. Green and red areas represent the normal ranges for healthy adult and pregnant women, respectively (0.85 to 2 ng/dL). Abbreviation: trim, trimester.

changing isoleucine 131 into threonine (p.I131T). The patient's mutation was inherited from his parents, who are both heterozygous carriers. The paternal grandmother and three siblings of the patient are heterozygotes for the mutation, whereas one of his brothers is a homozygous carrier [Fig. 1(a–c); Supplemental Fig. 2].

Targeted next-generation sequencing in the index case and his mother showed no pathogenic mutations in genes involved in thyroid hormonosynthesis, including *TG*, *TPO*, *NIS*, *DUOX2*, *DUOXA2*, *DEHAL1*, *TSHR*, and *GNAS* (data not shown).

## Functional characterization of the I131T-TRHR mutant

To determine whether the isoleucine-to-threonine mutation affected expression and trafficking of the receptor, the relative density of hemagglutinin-tagged receptors at the plasma membrane was quantified. Wild-type TRHR was strongly expressed on the plasma membrane, and there was no significant difference in receptor density when cells were transfected with equal amounts of complementary DNA encoding the I131T mutant or a 1:1 mixture of wild-type and mutant TRHR [Fig. 4(a)].

The molecular basis for the signaling defect in the I131T-TRHR was investigated by measuring the affinity of wild-type and mutant TRHR for a high-affinity, radiolabeled agonist, [$^3$H]Me-TRH. Cells were incubated with tracer [$^3$H]Me-TRH and different concentrations of unlabeled TRH under equilibrium conditions. Significantly higher concentrations of unlabeled TRH were required to decrease [$^3$H]Me-TRH binding to the I131T-TRHR, consistent with lower affinity for the natural

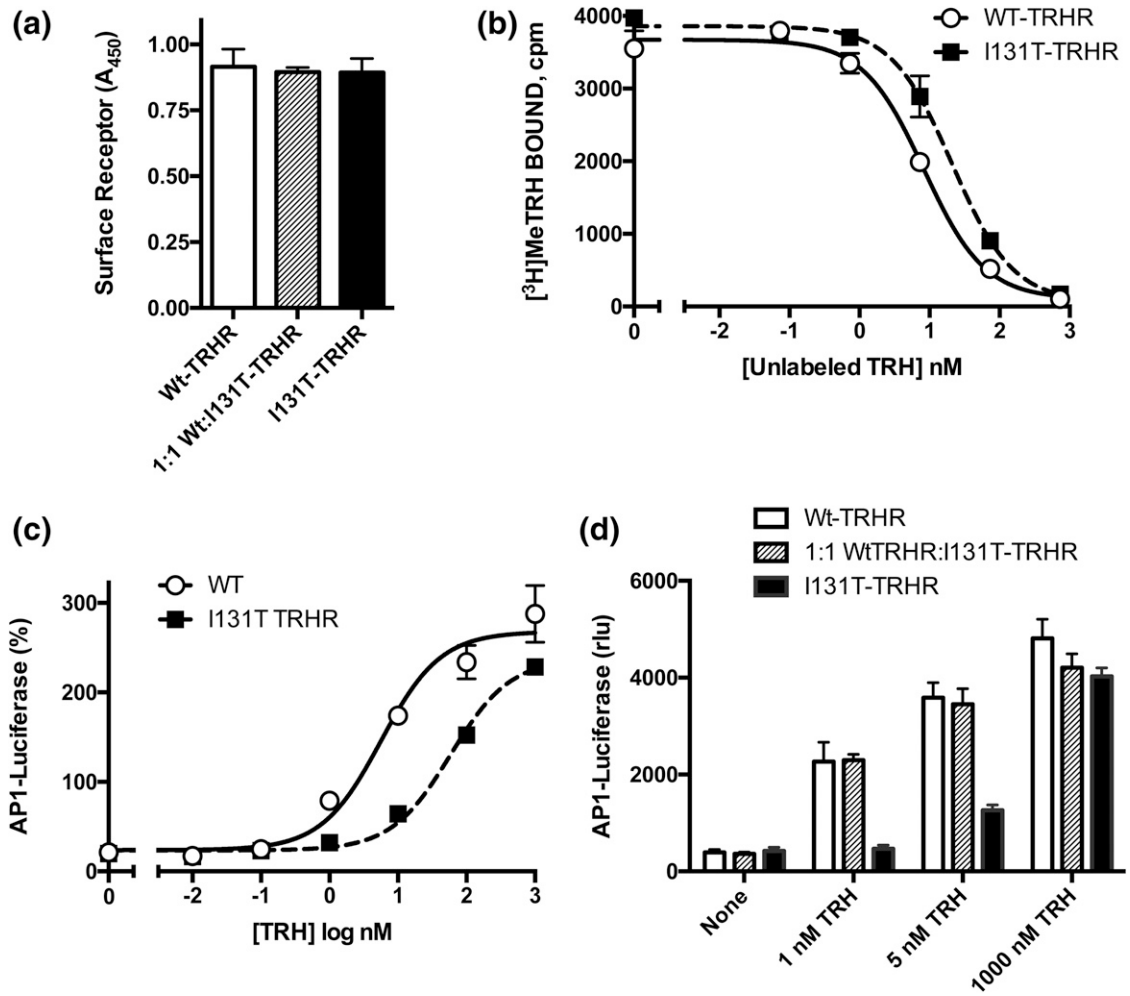



**Figure 4.** I131T-TRHR functional studies. (a) I131T-TRH receptors are located at the cell membrane at the same density as the wild type. Hemagglutinin-tagged TRH receptors on the cell surface were measured by enzyme-linked immunosorbent assay in the experiment depicted in panel b. (b) I131T-TRHR has reduced affinity for [$^3$H]Me-TRH. Cells transfected with control plasmid or TRH receptors were incubated with 2 nM [$^3$H]methyl-TRH and concentrations of unlabeled TRH shown, and specific binding was measured after 1.5 hours. (c, d) Cells were transfected to express wild-type and/or mutant TRH receptors and an AP1-luciferase reporter. After 24 hours, cells were stimulated for 4 hours with the concentrations of TRH shown, and luciferase activity was quantified. (c) Response to different concentrations of TRH showing that the I131T-TRHR has a higher half-maximal effective concentration but similar maximum response compared with the wild-type receptor. The M3 muscarinic receptor was cotransfected as a control for downstream effects; results are expressed as percentage of the response to 10 μM carbachol. (d) I131T- and wild-type (WT)-TRHR were transfected alone or together using a 1:1 DNA ratio and stimulated with the concentrations of TRH shown; luciferase activity is not normalized. Abbreviations: $A_{450}$, absorbance at 450 nm; rlu, relative light units.

ligand [Fig. 4(b)]. The relative affinity of the two receptors for TRH was 3.1 ± 0.3 and 9.1 ± 0.4 nM for wild-type and I131T mutant TRH receptors, respectively ($P < 0.05$).

The effect of the I131T mutation on signaling capacity was tested by expressing wild-type and mutant TRHR in HEK293 cells, a widely used system for evaluating GPCR signaling. TRHR signals through a classical Gq-coupled pathway, stimulating an increase in intracellular calcium and activation of PKC (26). Receptor activity was determined using an AP1-luciferase reporter containing a c-fos promoter sequence activated by the TRH-IP3-calcium-PKC pathway. TRH induced more than a 10-fold increase in AP1-luciferase activity in cells expressing the wild-type TRHR (average half-maximal effective concentration for TRH = 2.8 ± 0.9 nM [n = 7]). I131T-TRHR was capable of generating the same maximal response; however, significantly higher concentrations of TRH were required (average half-maximal effective concentration = 20.4 ± 0.8 nM [n = 6, $P < 0.05$ vs wild type]) [Fig. 4(c)].

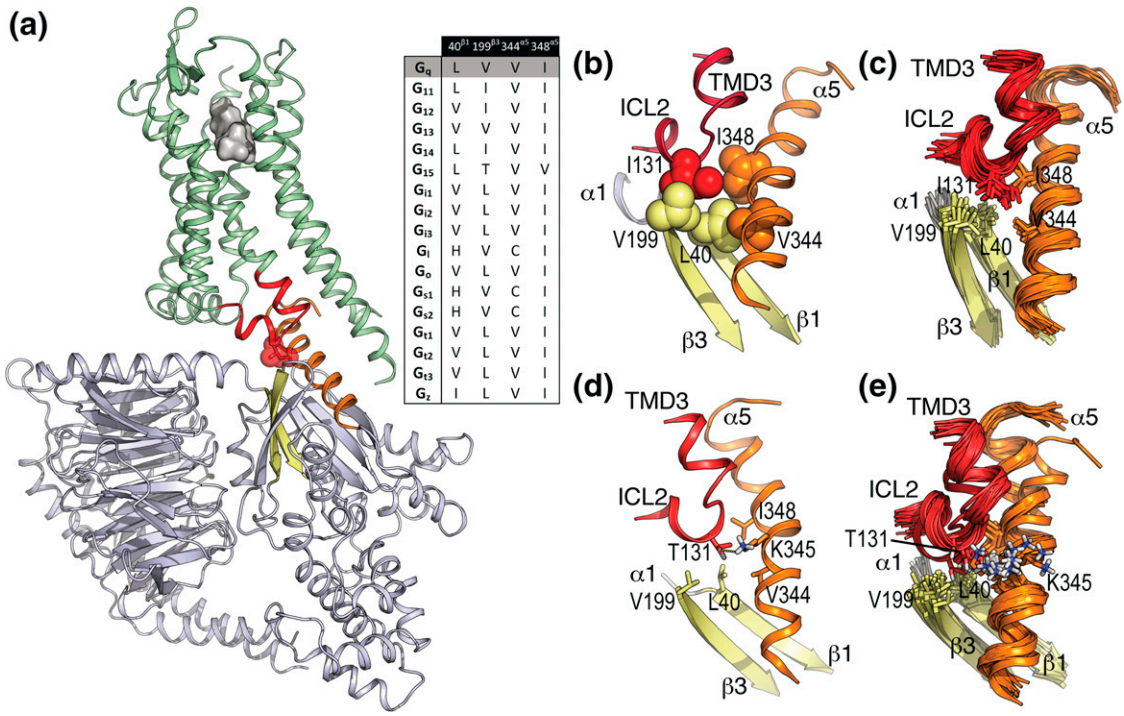
Signaling via the TRH receptor was also tested in cells coexpressing wild-type and I131T-TRHR mutant receptors in an attempt to mimic the situation in individuals heterozygous for the mutation. Because activity of the two receptors differed more at low concentrations of TRH, responses were determined either without any stimulus (to monitor constitutive activity), low TRH (1 and 5 nM), or maximally effective TRH (1 μM) [Fig. 4(d)]. As expected, the I131T-TRHR signaled weakly compared

with the wild type at 1 and 5 nM TRH [Fig. 4(d)]. At maximal TRH concentrations, no significant differences in activity between the mutant and wild type ($P > 0.05$) were present, consistent with the capacity of the mutant to generate maximal responses [Fig. 4(d)]. Cells coexpressing wild-type and mutant receptors responded as strongly as wild-type receptors. Constitutive activity was not affected by mutation of I131, indicating that functional impairment of I131T-TRHR involves ligand-activated signaling [Fig. 4(d)].

### I131T-TRHR mutation disrupts the interaction with Gq in the active-like TRH-TRHR-Gq model

An "active-like" model of the TRHR in complex with Gq was built [Fig. 5(a); Supplemental Methods]. $I131^{ICL2}$ is located in ICL 2 and pointing toward Gq. The molecular interface between TRHR and Gq is mainly formed by the interaction of TMDs 3, 5, and 6 of TRHR with the C-terminal $\alpha 5$ helix of Gq and ICL 2 of TRHR with the N-terminal $\alpha 1$ helix and $\beta 1$ and $\beta 3$ strands of Gq. In detail, the hydrophobic side chain of $I131^{ICL2}$ forms hydrophobic-hydrophobic interactions with $L40^{\beta 1}$ in the $\beta 1$ strand, $V199^{\beta 3}$ in the $\beta 3$ strand, and $V344^{\alpha 5}$ and $I348^{\alpha 5}$ in the $\alpha 5$ helix of Gq [Fig. 5(b)]. These interactions remain stable during MD simulation [see later and Fig. 5(c)]. Statistical analysis of sequence conservation at this $I131^{ICL2}$ position, in the nonolfactory class A GPCR superfamily, was performed in a refined multiple sequence alignment. Among the 287 sequences reported in GPCRdb (27), we selected 247 sequences that contain at least one of the two characteristic signatures of ICL 2: a Pro residue at position $(E/D)R^{3.50}YX_5P$ (28) that starts the two-turn $\alpha$-helix conformation of ICL 2 and a Tyr residue at position $(E/D)R^{3.50}YX_5PX_2\underline{Y}$ that interacts with the negative charge of the (E/D)RY motif in TMD3 (29). Clearly, this refined sequence alignment shows that most GPCRs contain a hydrophobic residue at the homologous $I131^{ICL2}$ position (L, 39%; F, 14%; V, 11%; I, 9%). Similar analysis of sequence conservation in the G-protein family, at the homologous $L40^{\beta 1}$, $V199^{\beta 3}$, $V344^{\alpha 5}$, and $I348^{\alpha 5}$ positions [inset of Fig. 5(a)], shows that this hydrophobic pocket is conserved but with some variation.

To evaluate the effect of the $I131^{ICL2}T$ mutation in the TRHR-Gq interface, we performed MD simulations of wild-type [Fig. 5(c)] and mutant [Fig. 5(d) and 5(e)] receptors of the "active-like" model of TRHR in complex with Gq (Supplemental Methods). Replacement of $I131^{ICL2}$ by Thr adds a polar hydroxyl group at this




| | 40$^{\beta 1}$ | 199$^{\beta 3}$ | 344$^{\alpha 5}$ | 348$^{\alpha 5}$ |
|---|---|---|---|---|
| $G_q$ | L | V | V | I |
| $G_{11}$ | L | I | V | I |
| $G_{12}$ | V | I | V | I |
| $G_{13}$ | V | V | V | I |
| $G_{14}$ | L | I | V | I |
| $G_{15}$ | L | T | V | V |
| $G_{i1}$ | V | L | V | I |
| $G_{i2}$ | V | L | V | I |
| $G_{i3}$ | V | L | V | I |
| $G_i$ | H | V | C | I |
| $G_o$ | V | L | V | I |
| $G_{s1}$ | H | V | C | I |
| $G_{s2}$ | H | V | C | I |
| $G_{t1}$ | V | L | V | I |
| $G_{t2}$ | V | L | V | I |
| $G_{t3}$ | V | L | V | I |
| $G_z$ | I | L | V | I |

**Figure 5.** Computational model of the TRH-TRHR-Gq complex. (a) General view of the "active-like" model of TRHR in complex with Gq at the intracellular site and TRH at the extracellular site. TRH is shown as a gray surface, TRHR is shown as ribbons in green (intracellular TMD 3 and ICL 2 are shown in red), Gq is shown in blue-white ($\beta 1$ and $\beta 3$ strands in yellow and the $\alpha 5$ helix in orange), and I113 is shown in red spheres. Statistical analysis of sequence conservation in the G-protein family at the homologous $L40^{\beta 1}$, $V199^{\beta 3}$, $V344^{\alpha 5}$, and $I348^{\alpha 5}$ positions of Gq. (b) Detailed view of the interaction of $I131^{ICL2}$ (wild-type) in ICL 2 of TRHR (in red) with $L40^{\beta 1}$ in the $\beta 1$ strand (in yellow), $V199^{\beta 3}$ in the $\beta 3$ strand (in yellow), and $V344^{\alpha 5}$ and $I348^{\alpha 5}$ in the $\alpha 5$ helix (in orange) of Gq. (c) Cartoon representation of 20 snapshots extracted from 500-ns MD trajectory of wild-type TRHR. The side chains of $I113^{ICL2}$, $L40^{\beta 1}$, $V199^{\beta 3}$, $V344^{\alpha 5}$, and $I348^{\alpha 5}$ are shown as sticks. (d) Detailed view of the interaction of $T131^{ICL2}$ (TRHR mutant) with $L40^{\beta 1}$, $V199^{\beta 3}$, $V344^{\alpha 5}$, $K345^{\alpha 5}$, and $I348^{\alpha 5}$ of Gq. (e) Cartoon representation of 20 snapshots extracted from 500-ns MD trajectory of $I131^{ICL2}T$ mutant receptor.

TRHR-Gq interface. However, this small change in polarity causes a significant distortion. Whereas the hydrophobic $I131^{ICL2}$ side chain maintains the interactions with $L40^{\beta 1}$, $V199^{\beta 3}$, $V344^{\alpha 5}$, and $I348^{\alpha 5}$ of Gq during the MD simulation [Fig. 5(c)], the polar side chain of $T131^{ICL2}$ partly modifies the interaction of the $\alpha 5$ helix with TRHR [Fig. 5(e)] to interact with the polar side chain of $K344^{\alpha 5}$ [Fig. 5(d)]. Therefore, insertion of a polar Thr side chain into this hydrophobic pocket disrupts TRHR-Gq coupling.

## Discussion

A unique missense *TRHR* mutation was identified in a consanguineous family causing central hypothyroidism in homozygotes and borderline and intermittent TSH elevation in heterozygous carriers of the defect. The study suggests that two affected *TRHR* alleles are necessary to develop the full hypothyroid phenotype and expands the scope of thyroid hormone derangements associated with TRHR mutations to include hyperthyrotropinemia, when one allele is affected.

Our index patient came to medical attention at the age of 8 for abnormal thyroid function tests in a routine checkup. Although the patient was overweight, features of hypothyroidism were not overt, but low FT4 and normal TSH suggested central hypothyroidism. Interestingly, 38% of children with central hypothyroidism due to *IGSF1* defects are overweight or obese (30).

Retrospectively, it was evident that the patient already showed subtle hypothyroidism and hyperthyrotropinemia at 6 years of age. Strikingly, the youngest sibling of the proband, also homozygous for the defect, shows isolated hyperthyrotropinemia at age of 5. This suggests that TSH elevation may precede the development of overt hypothyroidism in homozygotes and represents a compensatory state that eventually fails, along with increased demands for thyroid hormones.

In pregnancy, thyroid hormone requirements physiologically increase and (mild) defects of the thyroid hormone axis are transiently detectable (31, 32). The mother of our patient, a heterozygous carrier of I131T, became pregnant during the course of the study. Following the guidelines of the American Thyroid Association for thyroid dysfunction in pregnancy, her TSH was elevated during the first trimester (TSH: 3.72 mIU/L; Normal: <2.5 mIU/L) (23). L-T4 was instituted, but TSH elevation recurred in the third trimester. After delivery, the mother's TSH level returned to normal and L-T4 treatment was withdrawn. Therefore, gestational hyperthyrotropinemia in the pregnant mother may represent a compensatory attempt to meet increased demands for thyroid hormones during pregnancy.

The functional differences between the unique I131T mutation and the previously described TRHR mutations correlate well with the signaling capacity of the affected receptors (6–8). The R17X, S115-T117del+A118T, and P81R mutations all showed deleterious effects on receptor function. R17X results in a prematurely truncated protein, missing all seven TMDs. S115-T117del+A118T deeply alters the tertiary structure of the TMD 3 (essential to receptor function), and P81R allegedly alters the conformation of TMD 2 and therefore the TRH binding pocket. Unlike previous cases with biallelic TRHR mutations, our patient showed normal TSH and PRL responses to TRH (6, 7). The normal TSH responses of our patients to standard dynamic TRH tests (involving *in vivo* administration of high doses of TRH) are consistent with the finding that wild-type and I131T-TRHRs respond identically to saturating concentrations of TRH *in vitro*.

I131T is the first missense mutation identified in the ICL 2 of the TRHR and is located at a highly conserved hydrophobic position of the class-A GPCRs at the (E/D) $R^{3.50}YX_5P\Phi XY$ motif, which is important for the essential conformational changes required for G-protein activation (29). Unlike the three previously identified mutations in TRHR that severely impair TRH binding and signal transduction (6–8), the unique I131T-TRHR mutant caused a threefold reduction in TRH affinity and a 7.3-fold shift in the concentration-response curve for activation of the Gq-IP3-PKC pathway yet showed normal responses to high concentrations of TRH, which might relate with the milder phenotype of hypothyroidism in the family. The model of TRHR in complex with Gq (Fig. 5) shows that the hydrophobic $I131^{ICL2}$ side chain of TRHR interacts with a hydrophobic pocket formed by $L40^{\beta 1}$, $V199^{\beta 3}$, $V344^{\alpha 5}$, and $I348^{\alpha 5}$ of Gq. Thus, it seems reasonable to propose that insertion of a polar Thr side chain into this hydrophobic pocket of Gq disrupts TRHR-Gq coupling. Accordingly, higher concentrations of TRH are necessary to achieve the same maximal response in the I131T mutant [Fig. 4(c)]. Mutation of this hydrophobic residue in other GPCRs has similar effects but with a different extent of G-protein uncoupling (33–37).

The mechanism by which the $I131^{ICL2}T$ mutation at the intracellular site influences the binding of TRH to TRHR at the extracellular site is clearly indirect. Two related mechanisms have been proposed to explain this effect. First, the constitutive activity of the receptor (the equilibrium between inactive and active states in the absence of ligand) modulates ligand affinity and selectivity (38). Second, there is an allosteric coupling from G-protein to the agonist-binding pocket in GPCRs (39). Because the I131T mutation does not influence the

constitutive activity of TRHR [Fig. 4(d)], we suggest that uncoupling of Gq from I131T-TRHR at the intracellular site causes the decrease of TRH binding to TRHR at the extracellular site [Fig. 4(b)]. This work provides a structural explanation for the G-protein–mediated enhancement of agonist affinity.

In addition to *TSHB* transcription, TRHR regulates glycosylation of TSH, which is required for the full biopotency of the TSH dimer (13, 14). Patients with central hypothyroidism, especially of hypothalamic origin, have been described with low bioactive TSH and sometimes slightly elevated immunoreactive TSH (1). The I131T-TRHR mutant seems to hinder the expected increase of FT4 after TRH stimulation *in vivo*, suggesting impaired TSH bioactivity. As reported in $Trh^{-/-}$ knockout mice (and also $Trh^{-/+}$ mice to a lesser extent), decreased TRH-TRHR signaling in thyrotropes might be responsible for the development of hyperthyrotropinemia with low TSH biopotency in carriers of I131T (40). In the absence of TRH action, hyperthyrotropinemia exhibited by *Trh*-deficient mice is explained by decreased negative feedback of thyroid hormones at the pituitary, leading to increased synthesis of a TSH with low biological activity (40). We propose a similar situation in human pituitaries with a partially defective I131T-TRHR mutant.

The intrinsic mechanism of hyperthyrotropinemia in this family is not known, but it may relate to the uniquely milder functional behavior of the I131T-TRHR mutant itself, because a primary thyroidal defect was ruled out in this family and obesity in some (but not all) individuals of the pedigree is not linked to this feature. However, TSH elevation in this family contrasts with the normal TSH levels observed in knockout mice with deletion of the *Trhr1* gene ($Trhr1^{-/-}$) (41) and in individuals with previously described mutations in the *TRHR* gene (6–8). Two significant differences may exert a role in hyperthyrotropinemia detected in patients with I131T-TRHR mutation. First, as shown, the I131T mutant protein is stable and normally reaches the membrane. In contrast, previously described mutations in *TRHR* are reported to have no residual activity. Likewise, *Trhr1* knockout mice are engineered to have complete deletion of the gene, leading to the absence of TRH receptors at the thyrotropic cell membrane. Second, the I131T mutant has a subtle effect on TRHR function, which can be rescued by increased TRH levels.

In summary, we identified a unique missense mutation (p.I131T) in *TRHR* associated with overt central hypothyroidism in the biallelic state. Although inheritance of TRHR defects is typically recessive, we describe the presence of central hyperthyrotropinemia in heterozygous carriers of this mutation. Hyperthyrotropinemia is proposed as a compensatory state preceding hypothyroidism in homozygotes. In individuals heterozygous for I131T-TRHR, central hyperthyrotropinemia is present with normal T4. However, development of hypothyroidism during pregnancy should be specifically ruled out. Undiagnosed central hypothyroidism in children calls for a higher degree of suspicion from pediatric endocrinologists dealing with patients with borderline-low T4 and normal TSH or with isolated hyperthyrotropinemia due to thyrotropic failure (22).

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# CAPÍTULO V

## Hipotiroidismo central, pérdida auditiva, malformación de Arnold Chiari tipo I y transtorno de déficit de atención e hiperactividad (TDAH) en un paciente con una inactivación en hemicigosis del gen *TBL1X*

*TBL1X* codifica una subunidad esencial del complejo NCOR-SMRT, implicado en la represión de las acciones de la hormona tiroidea a través de su receptor. *TBL1X* está altamente expresado en la hipófisis y también en el hipotálamo. Los defectos en este gen se han asociado recientemente con hipotiroidismo central y pérdida auditiva.

El objetivo de este trabajó consistió en la caracterización clínica y genética de un paciente con hipotiroidismo central causado por una mutación de codón de parada en el gen *TBL1X*.
Se identificó la mutación de codón de parada p.R339X en hemicigosis en el gen *TBL1X* en un paciente diagnosticado de hipotiroidismo central a los 6 años de edad (FT4: 10,42, Normal: 12-22 pmol/L, TSH: 1,57, Normal: 0,7-5,7 mIU/L) con una respuesta de TSH ligeramente reducida en el test de TRH y episodios de encopresis y estreñimiento. A los 7 años de edad, la ecografía tiroidea mostró una hipoplasia leve, la RM del cerebro mostró una hipófisis normal en tamaño y localización, sin embargo, el paciente presentó una malformación de Arnold-Chiari Tipo I. El paciente fue diagnosticado con trastorno por déficit de atención e hiperactividad (TDAH). A los 10 años de edad, por audiometría se identificó una deficiencia en la conducción aérea a altas frecuencias. La presencia de un codón de parada prematuro en la proteína mutada causó la pérdida de seis de ocho dominios de repetición WD-40, que están implicados en las interaccione proteína-proteína.

En conclusión, se ha identificado la primera mutación de codón de parada en el gen *TBL1X*, en un paciente con hipotiroidismo central y pérdida de audición y por primera vez, con TDAH, encopresis y malformación de Arnold-Chiari tipo I. Dada la importancia de TBL1X para la activación de múltiples vías de señalización intracelular en diferentes tejidos del organismo (cerebro, cerebelo, cóclea, hipófisis), no es sorprendente que las mutaciones en este gen conduzcan a características fenotípicas más extensas que las descritas hasta la fecha y que podrían variar dependiendo de la gravedad de la mutación.

# Central hypothyroidism, hearing loss, encopresis, Chiari I malformation and attention deficit-hyperactivity disorder (ADHD) in a patient with hemizygous inactivation of *TBL1X*

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*Abbreviated Title: Human phenotype of the hemizygous inactivation of TBL1X.*

*Key terms:* central hypothyroidism, pituitary, attention deficit hyperactivity disorder (ADHD), Arnold Chiari type I, hearing loss, *TBL1X* gene

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Context: *TBL1X* gene encodes a subunit of the NCOR-SMRT complex involves in repression of thyroid hormone actions. *TBL1X* is highly expressed in pituitary and also in hypothalamus. Defects in this gene are recently associated with central hypothyroidism and hearing loss.

Objective: Clinical and genetic characterization of a patient with central hypothyroidism caused by *TBL1X* non-sense mutation.

Methods: Patient and his relatives were clinically phenotyped with thyroid hormone profile, TRH test, brain MRI, thyroid ultrasounds, audiometry and psychological evaluation. Candidate *TRH*, *TRHR*, *TSHB* and *IGSF1* genes were screened for mutations. A panel of 390 genes involves in primary and central hypothyroidism (including TBL1X) was studied in the index case.

Results: A non-sense hemizygous mutation (p.R339X) was identified in *TBL1X* gene in a patient diagnosed of central hypothyroidism at 6 years of age (FT4: 10.42, Normal: 12-22 pmol/L; TSH: 1.57, Normal: 0.7-5.7 mIU/L) with mildly reduced TSH response in the TRH test and encopresis and constipation episodes. At 7 years of age thyroid ultrasound showed a mild hypoplasia, brain MRI showed normal pituitary size and shape, however an Arnold-Chiari type I malformation. Patient was diagnosed with attention-deficit hyperactivity disorder (ADHD). At 10 years of age, a pure tone audiometry (PTA) identified poor hearing thresholds in the air conduction at high frequencies. The presence of a premature stop codon in the mutated protein caused the loss of six to eight WD-40 repeat domains, which are involved in protein interactions.

Conclusion: We identified the first non-sense mutation in the *TBL1X* gene in a patient with central hypothyroidism and hearing loss and for the first time, with ADHD, encopresis and Arnold-Chiari malformation. Given the importance of *TBL1X* for activation of multiple intracellular signaling pathways in different tissues of the organism (brain, cerebellum, cochlea, hypophysis), it is not surprising that mutations in this gene lead to phenotypic characteristics more extensive than those previously described and which may vary depending on the severity of mutation.

## Introduction

Central congenital hypothyroidism (CCH) is an underdiagnosed disease characterized by deficient production of thyroid hormones due to reduced TSH synthesis, secretion or bioactivity, which fails to properly stimulate an otherwise normal thyroid gland (Persani L, 2012; García M *et al*., 2014, Annex I of this Thesis). To date, defects in only four genes have been identified in patients with CCH: *TSHB* (encoding the B-subunit of the TSH glycoprotein hormone), *TRHR* (the specific 7-transmembrane domain receptor for hypothalamic TRH), *IGSF1* (a protein regulating the expression of TRHR in pituitary thyrotropes), and the recently identified *TBL1X* (a subunit of the NCoR-SMRT complex) gene (García M *et al*., 2014; Heinen CA *et al*., 2016, Annex I of this Thesis).

*TBL1X* is located at the Xp22.3-p22.2 chromosome and encodes a protein highly expressed in pituitary and present in hypothalamus, representing an essential subunit of the nuclear receptor corepressor (NCoR)-silencing mediator complex for retinoid and thyroid hormones receptors (SMRT) involved in T3-regulated gene expression (Yoon HG et al., 2003; Astapova I *et al*., 2011; Heinen CA *et al*., 2016).

A recent publication reported the identification of (inherited) missense and splice-site point mutations in the human *TBL1X* gene in six unrelated families, variably presentingT4 deficiency or normal T4 levels at birth (Heinen CA *et al*., 2016). Defects in this gene also associate hearing loss (Bassi MT *et al*., 1999; Heinen CA *et al*., 2016). Neurodevelopmental defects were not reported in children or adults of such pedigrees. The pathogenic mechanism underlying this X-linked disorder remains unknown.

Here, we present the first *nonsense* and *de novo* mutation in the *TBL1X* gene representing the full inactivation of the gene, in a patient with CCH, hearing loss, attention-deficit and hyperactivity disorder (ADHD) and Chiari type I malformation.

## Materials and Methods

Informed consent for genetic studies was obtained from index patient and his family, according to protocols in our Institution.

The coding regions of the candidate genes for central congenital hypothyroidism *TSHB*, *TRHR* and *IGSF1*were amplified by PCR using appropriate primers flanking each exon. PCR products were purified and directly sequenced on an automated DNA sequencer (3100 Genetic Analyzer, Applied Biosystems). A targeted panel of Next Generation Sequencing (NGS) including the coding region and the flanking intronic boundaries of 390genes involved in

thyroid physiology were investigated using NextSeq 500 (Illumina, San Diego, California, EEUU) platform. Genetic variants were filtered on population frequency and pathogenic prediction criteria using 5 publically available databases (dbSNP, 1000 Genomes Project, The Exome Aggregation Consortium, Human Gene Mutation Database and ClinVar) and the current scientific literature. The presence of the mutation identified in the index case was investigated in patient´s parents and sister by Sanger sequencing.

The patient was clinically (hormonally, morphologically) and genetic characterized. Serum TSH, Free thyroxine (FT4), Total triiodothyronine (TT3), prolactin and cortisol were determined with Quimioluminiscent immunoanalysis in Centaur XP (Siemens, Healthineers). IGF1, IGFBP3 and ACTH were measured with Quimioluminiscent immunoanalysis in Immulite 2000 (Siemens, Healthineers). TRH stimulation test was performed as previously reported (Van Tijn DA *et al*., 2008B). Thyroid volume was calculated from three dimensional measurements of each lobule by ultrasounds, according to the formula V (ml) = 0.479 x depth x width x length (cm) and compared with a local population (García-Ascaso, MT, 2013).

## Results

### *Clinical Case*

The patient is a 10 year old male of Spanish descent, born to non-consanguineous parents in a twin pregnancy derived from *in vitro* fertilization (IVF) of own parental sperm and oocytes (Figure 1A).

He was not detected by the TSH-based Neonatal Screening Program for Congenital Hypothyroidism, showed unremarkable weight, growth and neurological development in infancy. At five years of age he showed encopresis and constipation treated with macrogol. At six years he was referred to Pediatric Endocrinology for evaluation of abnormal thyroid function tests with low free T4 (10.16 pmol/L, N: 12-22 pmol/L) but normal TSH (1.06 mIU/L, N: 0.7-5.7 mIU/L) consistent with central hypothyroidism (Table 1).Total T3 was normal (1.46 ng/ml, N: 0.8-2 ng/ml) (Table 1). Anti-thyroglobulin antibodies were negative (18.6 IU/m, N: 0-115 IU/m), ruling out autoimmune thyroid disease. A180 min-TRH test was performed to investigate the origin of his hypothyroidism, showing reduced TSH stimulation (low TSH capacity) and full-recovery of basal TSH levels at 180 minutes (Figure 1B), compatible with a pituitary defect. Moreover, his TSH-FT4 relation was studied in the model of Dietrich *et al* (Dietrich JW *et al*., 2012), and despite of the first determination fall inside the green area representing the normal dynamic relation, the other two posterior determinations fall outside the normality, representing a possible thyrotropic failure (Figure 1C).He did not have clear

symptoms of hypothyroidism and presented acceptable school performance with help of additional classes, being diagnosed with attention deficit disorder. Levo-thyroxine (L-T4) replacement was started at a dose of 1.1 µg/Kg/day, which normalized his FT4 levels (15.70 pmol/L) at the expense of reducing TSH (0.37 mIU/L) (Table 1), characteristic of central hypothyroidism. At 7 years of age and one month after treatment was started, thyroid ultrasound showed a mild hypoplasia of the thyroid gland (1.84 ml, p3: 1.61 ml, p50: 3.05 ml, 8) (Figure 1D). No defect in any other pituitary axes was identified, with basal cortisol, ACTH, IGF1, IGFBP3, FSH, LH and testosterone within the normal ranges (Table 1). Brain MRI showed normal pituitary size and shape, however an Arnold-Chiari type I malformation was detected (Figure 1E).At 7 years of age the patient was formally diagnosed with attention-deficit hyperactivity disorder (ADHD).He was not treated for ADHD based on parental decision. At 10 year of age, a pure tone audiometry (PTA) was performed identifying poor hearing thresholds in the air conduction at high frequencies compared with the age-specific reference interval (Rodríguez Valiente A *et al*., 2015) (Figure 1F). In the last clinical visit at 10 years of age, his height (141.4 cm (p57), 0.19 SD), and weight (35.2 Kg (p45) -0.15 SD, BMI 17.61 (p40) -0.27 SD) were normal, as was his gonadal development with bilateral testicular volumes of 3 ml in accordance with pubertal Tanner I stage.

His father presents type I diabetes mellitus (DM1) and familiar history of type 2 DM (DM2), but no antecedents of hypothyroidism. His mother and twin sister showed unremarkable phenotype (Figure 1A).

***Identification of the TBL1X mutation***

Molecular analysis for mutations by Sanger sequencing of the 3 candidate genes associated with central hypothyroidism (TRHR, TSHB and IGSF1) and other 7 genes related with regulation of hypothalamus-pituitary axis (TRH, LHX3, LHX4, SOX3, GATA2, SF1 and NR4A1) do not reveal any defects in the patient. Targeted NGS of a panel of genes including candidates for central and primary hypothyroidism identified a rare non-sense hemizygous mutation in exon 11 of the *TBL1X* gene (c.1015C>T) changing Arginine 339 into Stop codon (p.R339X) (transcript accession number NM_001139466.1). The mutation was confirmed by Sanger sequencing and segregated in the family. The p.R339Xis a *de novo* mutation since it was not identified in parents or sister of the patient (Figure 1A and Figure 2A). This variant was not reported in databases for genomic variants.

TBL1X contains an N-terminal LisH domain, a putative F Box domain for oligomerization and a C-terminal WD40 domain with 8 WD-repeats base for interaction with other nuclear proteins,

including nuclear translocation factors (Yoon HG *et al*., 2003).The amino acid 339 forms part of the highly conserved WD40 repeat domain of TBL1X (WD 3) and the mutations prematurely terminates removes most of the domain (Figure 2B).

## Discussion

TBL1X is an essential subunit of the nuclear receptor co-repressor (NCoR) and silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) co-repressor complexes that regulate transcription of many different genes (Perissi V *et al*., 2010). The function of these co-repressor complexes is exerted through remodelling of nuclear chromatin by histone-deacetylase 3 (HDA3) and interaction with classical DNA-binding nuclear receptors at the promoter of target genes (Figure 2). Importantly, NCoR-SMRT regulates T3-dependent gene expression through the thyroid hormone receptors (TR) and retinoc acid receptors (RAR, RXR) (Yoon HG *et al*., 2003). TBL1X together with its receptor (TBL1XR1) also regulate B catenin-mediated Wnt-responsive genes, controlling cell proliferation and cell fate (MacDonald BT *et al*., 2009; Choi HK *et al*., 2011). Finally, NCoR-SMRT co-repressor complexes are also involved in synaptogenesis and synaptic activity and homeostasis (Bourgeron T, 2015).

Given the importance of TBL1X for activation of multiple intracellular signalling pathways, it is surprising that mutations or deletions so far known in the gene lead to restricted/limited phenotypes of deafness and central hypothyroidism. In the present study we describe the first *de novo,* early stop-codon mutation in *TBL1X* in a patient with a wider clinical phenotype including central hypothyroidism, hearing loss, attention-deficit hyperactivity disorder and learning difficulties, encopresis and Arnold-Chiari type I malformation at the skull base.

***Central congenital hypothyroidism*** is an underdiagnosed disease. The majority of cases are detected at peri-pubertal ages or in adulthood, since the TSH-based neonatal screening used in the most countries cannot detect defects in the hypothalamus-pituitary axis by the presence of normal TSH values (Persani L, 2012).

The first patients reported with *TBL1X* mutations (4/9) were detected in the T4-based neonatal screening program in Netherlands. Defects in this gene may cause variable hypothyroxinemia but sometimes normal T4 levels (Heinen CA *et al*., 2016). Our patient was not detected at a TSH-based CH screening and was diagnosed the age of 6 years by abnormal thyroid profile in the study of encopresis and constipation, not showing clear symptoms of hypothyroidism. His TRH test showed a decreased TSH secretion but with normal dynamycs. These results contrast

with the normal TRH test showed in patients previously reported, probably due to mutations in these patients caused a milder defect in *TBL1X* (Heinen CA *et al*., 2016). Moreover, our patient present mild thyroid hypoplasia, similar to patients previously described with TBL1X defects as well as TSHB and IGSF1 defects (Bonomi M *et al*., 2001; Heinen CA *et al*., 2016; Joustra SD *et al*., 2016A). This reduction of thyroid gland volume may be attributed to the lower TSH stimulation of the thyroid gland.

*TSHB* promoter is negatively regulated by T3 in through ligand-dependent repression exerted by TR. Since *TBL1X* defects show low-T4, this suggests that TBL1Xis physiologically involved in the enhancement of *TSHB* transcription in response to low T3 in thyrotropes, leading shortage of TSH secretion, and not sufficient stimulation of the thyroid (Figure 2C). TBL1X is also expressed in the parvocellular neurons of the paraventricular nucleus (PVN) of the hypothalamus suggesting coexpression with TRH (Heinen CA *et al*., 2016). Therefore, it could be speculated that TBL1X-depednet hypothyroidism could me mechanistically mediated to defective feed-back loop of low T3 at the pituitary, but also an intrinsic defect of TSHB transcription by low TRH-TSHR signalling.

***Sensorineural hearing loss*** was specifically searched for during the course of the study, once the gene defect was identified. The defect was bilateral and mild, being restricted to high tones and so far not involving clinically relevant disability. Follow-up of his audition capacity is warranted since deafness related to TBL1X deletions seems to progress with age (Bassi MT *et al*., 1999).

Thyroid hormone plays an essential role in development and differentiation of the inner ear, participating in its maturation from early stages of fetal development (Ng L *et al*., 2013). Effects of thyroid hormone are mediated by its receptors (TRs). T3 binds to TRs inducing conformational changes that cause the exchange of transcriptional co-repressors or co-activators, including *TBL1X* (Figure 2D). Thrb-deficient mice are deaf and have multiple morphological defects in the cochlea, a phenotype similar to that observed in humans with genetic defects in *TBLX1* (Brucker-Davis F *et al*., 1996; Griffith AJ *et al*., 2002). On the other hand, *TBL1X* is a subunit of the NCOR-SMRT co-repressor complex of T3-TRs, and it has been described that defects in *TBL1X* are associated with deafness in humans, representing a phenocopy of THRB defects in humans (Guenther MG *et al*., 2000). Therefore, it is likely that auditory defects related to TBLX1 deficiency could be mediated by dysregulation of THRB expression in the inner ear (Bassi MT *et al*., 1999; Heinen CA *et al*., 2016) (Figure 2D).

***Attention deficit and hyperactivity disorder*** (ADHD), and ***learning difficulties*** at school were revealed at 6 years of age in the course of the wider study. Following DSM-V, these neurodevelopmental features fall in the scope of Autism spectrum disorders (ASD) (Huguet G *et al*., 2013).

ASD has been consistently linked to defects in synaptogenesis and synapsis homeostasis (Bourgeron T, 2015). The NCoR-SMRT co-repressor complexes, including TBL1X, are known to regulate transcription of genes related to ASD, like *BDNF* (brain-derived neurotrophic factor), or Wnt target genes like CCND1 or ASCL2 (Bourgeron T, 2015), all important for synaptic homeostasis (Figure 2E). Interestingly, polymorphisms in TBL1X were associated to ASD in a GWAS study (Chung RH *et al*., 2011), but neurodevelopmental defects were not reported in patients with central hypothyroidism so far (Heinen CA *et al*., 2016). It is tempting to speculate that such patients, harbouring missense mutations in the gene may have residual function on target gene transcription, leading to absence or milder neurodevelopmental phenotypes, which may had not been evitable by a patient with a severely truncating mutant, as we describe in this paper. Alternatively, ASD features may appear specifically in mutations with dominant negative action over partners in the NCoR complex.

***Encopresis*** or faecal incontinence is a disabling disorder derived from alterations of motility in the gastrointestinal (GI) tract, especially the colonic part, which may alternate with constipation episodes. The pathogenesis is not well understood, but genetic factors were proposed (Johnston BD & Wright JA, 1993; Mikkelsen EJ, 2001). Strikingly, encopresis is very prevalent in patients with neurodevelopmental disorders like ADHD, or autism (Yektas C *et al*., 2016). Motility of the GI tract is performed by the smooth muscle cells but governed by the Enteric Neuron System (ENS).

NCoR-SMRT complex system is important for synaptic development and plasticity of ENS neurons, as it in the brain neurons. MECP2, another integral component of the NCoR-SMRT, is highly expressed in ENS where it regulates intestinal motility (Wahba G *et al*., 2016). Mutations in MECP2 cause the Rett Syndrome, a neurological disorder characterized by severe cognitive impairment, motor dyspraxia and seizures, but also (GI) dysmotility. Therefore it is tempting to speculate that encopretic/constipation symptoms in our patient relate to dysregulation of ENS gene transcription involved in normal GI motility.

***Chiari type I malformation*** (CMI) is produced when tonsils of the cerebellum locate below the foramen magnum of the skull, leading to a variety of neurological symptoms. In rodent,

thyroid hormone plays a crucial role in cerebellar development through its receptors. Deficiency of T3 results in abnormal cerebellar growth and differentiation. TRs and also those cofactor and other nuclear receptors, as corepressor complex (of which TBL1X is part), may play a role in regulating thyroid hormone sensitivity in the developing cerebellum (Koibuchi N, 2008) (Figure 2F). Tonsilar herniation is suspected to have a genetic basis since familial clustering is reported, and these associated genes could be regulated by T3. (Schanker BD *et al*., 2011). Our patient presented relative macrocephaly (head size within normal range, but disproportionately larger head related to height/weight centiles of the child) and he complained of headaches, especially after the practice of sports or a demanding exercise.

Wnt-beta catenin signalling contributes to early brain development (Bourgeron T, 2015). Overexpression of *CTNNB1* (gene for B catenin) leads to larger head size whereas defects in *CDH8*, a negative regulator of Wnt signalling, also leads to macrocephaly. As said above, Catenin-based regulation of transcription is governed by since TLB1X-TBL1XR1 are negative regulators of B-Catenin, it is tempting to speculate that a mutation in *TBL1X* is enhancing transcription of brain growth genes by B-catening, leading to increased intracranial pressure and CMI in our patient.

In summary, we described the first severely truncated *TBL1X* mutant identified in a male patient with central hypothyroidism associated with ADHD and Arnold-Chiari type I malformation.

**Acknowledgments**

We thank the patient and his family for their close collaboration and Mrs. Mercedes Tanarro for their expert technical assistance.

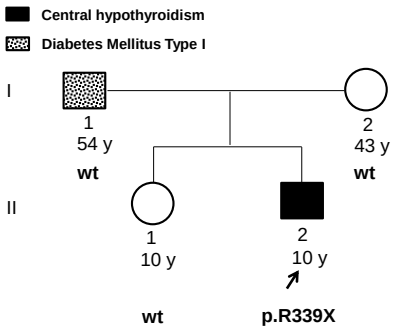
**Precis**

A first non-sense TBL1X mutation was identified in a patient with central hypothyroidism, mild thyroid hypoplasia, attention-deficit hyperactivity disorder, Arnold-Chiari type I malformation and loss of high hearing frequencies.

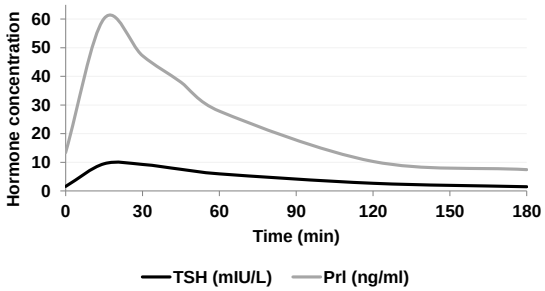
| Age | 5.5y | 6.25y | 6.3y* | 6.75y | 7y | 7.75y | 8.3y | 8.8y | 9.3y | 10y |
|---|---|---|---|---|---|---|---|---|---|---|
| **TSH** *mIU/L* (0.7-5.7) | 1.69 | 1.06 | 1.57 | **0.37** | **0.53** | **0.66** | 1.17 | **0.66** | 1.05 | 0.73 |
| **FT4** *pmol/L* (12-22) | **11.58** | **10.16** | **10.42** | 15.70 | 14.70 | 14.54 | 12.48 | 12.09 | **11.60** | 13.5 |
| **TT3** *ng/ml* (0.8-2) | - | - | 1.46 | 1.16 | - | - | - | - | - | 1.08 |
| **L-T4 dose** *µg/Kg/day* | - | - | - | 1.1 | 1.25 | 1.18 | 1.18 | 1.3 | 1.35 | 1.3 |
| **ACTH** *pg/ml* (5-46) | - | - | 13 | - | - | - | - | - | 28 | - |
| **Cortisol** *µg/dl* (5-25) | - | - | 9.9 | - | - | - | - | - | 21.3 | - |
| **IGF1** *ng/ml* | - | - | 179 | - | - | - | - | - | 210 | - |
| **IGF1BP3** *µg/ml* | - | - | 4 | - | - | - | - | - | 4.06 | - |
| **FSH** *mIU/ml* (1.5-12.4) | - | - | **1.45** | - | - | - | - | - | 3.73 | - |
| **LH** *mIU/ml* (1.7-8.6) | - | - | **<0.1** | - | - | - | - | - | **0.11** | - |
| **Testosterone** *ng/ml* (1.8-15.9) | - | - | **0.02** | - | - | - | - | - | **<0.1** | - |

**Table 1: Long-term follow up of thyroid and other pituitary axes profiles of the patient with TBL1X defect.** Hormone parameters are represented in chronological order along patient's life. In blue are represented hormone values below normal ranges. * Age at clinical diagnosis of the patient, when the TRH test was performed. TSH: thyrotropin, FT4: free thyroxine, TT3: total triiodothyronine, L-T4: levothyroxine treatment, ACTH: adrenocorticotrophic hormone, IGF1: insulin-like growth factor 1, IGF1BP3: insulin-like growth factor binding protein 3, FSH: follicle-stimulating hormone, LH: luteinizing hormone.

A



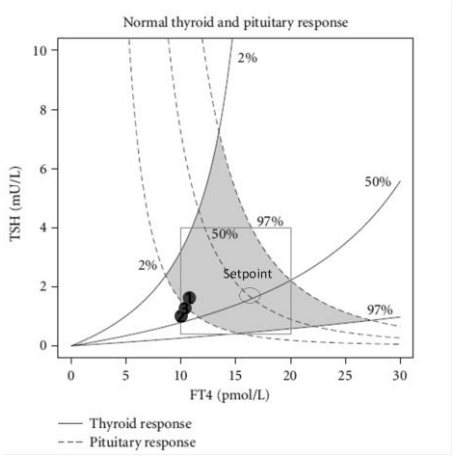

B




| Case | I.1 | II.1 | II.2 | I.2 | N.R. |
|---|---|---|---|---|---|
| **TSH** *mU/L* | 1.56 | 1.87 | 0.73 | 1.02 | 0.7-5.7 |
| **FT4** *pmol/L* | 13.9 | 13.1 | 13.5 | 14.4 | 12-22 |
| **LT4** *µg/Kg/day* | - | - | 1.3 | - | - |
| **HbA1C** *%* | **7** | 5.3 | 5.5 | 5.1 | 4-5.7 |
| **HbA1C** *nmol/mol* | **53** | 34.4 | 36.6 | 32.2 | 20-42 |

| Time (min) | 0 | 15 | 30 | 60 | 120 | 180 | %FT4 Incr. |
|---|---|---|---|---|---|---|---|
| TSH mU/L | 1.6 | 9.5 | 9.2 | 6.0 | 2.7 | 1.5 | |
| Fold-increase | 1.0 | 5.9 | 5.7 | 3.7 | 1.7 | 0.9 | |
| FT4 pmol/L (12-22) | *10.4* | - | - | - | - | 13.2 | 27% |

C



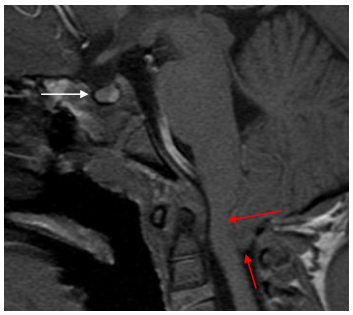

D

Right thyroid lobe
(0.8 x 2.3 x 1.1 cm)

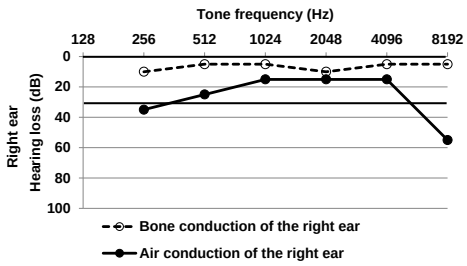
Left thyroid lobe
(0.8 x 2.3 x 1 cm)

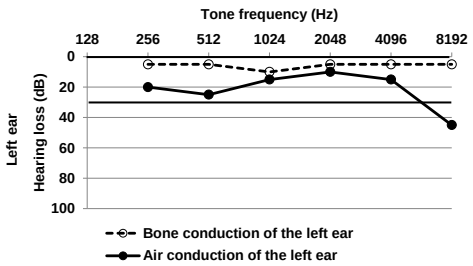
E

Brain MRI



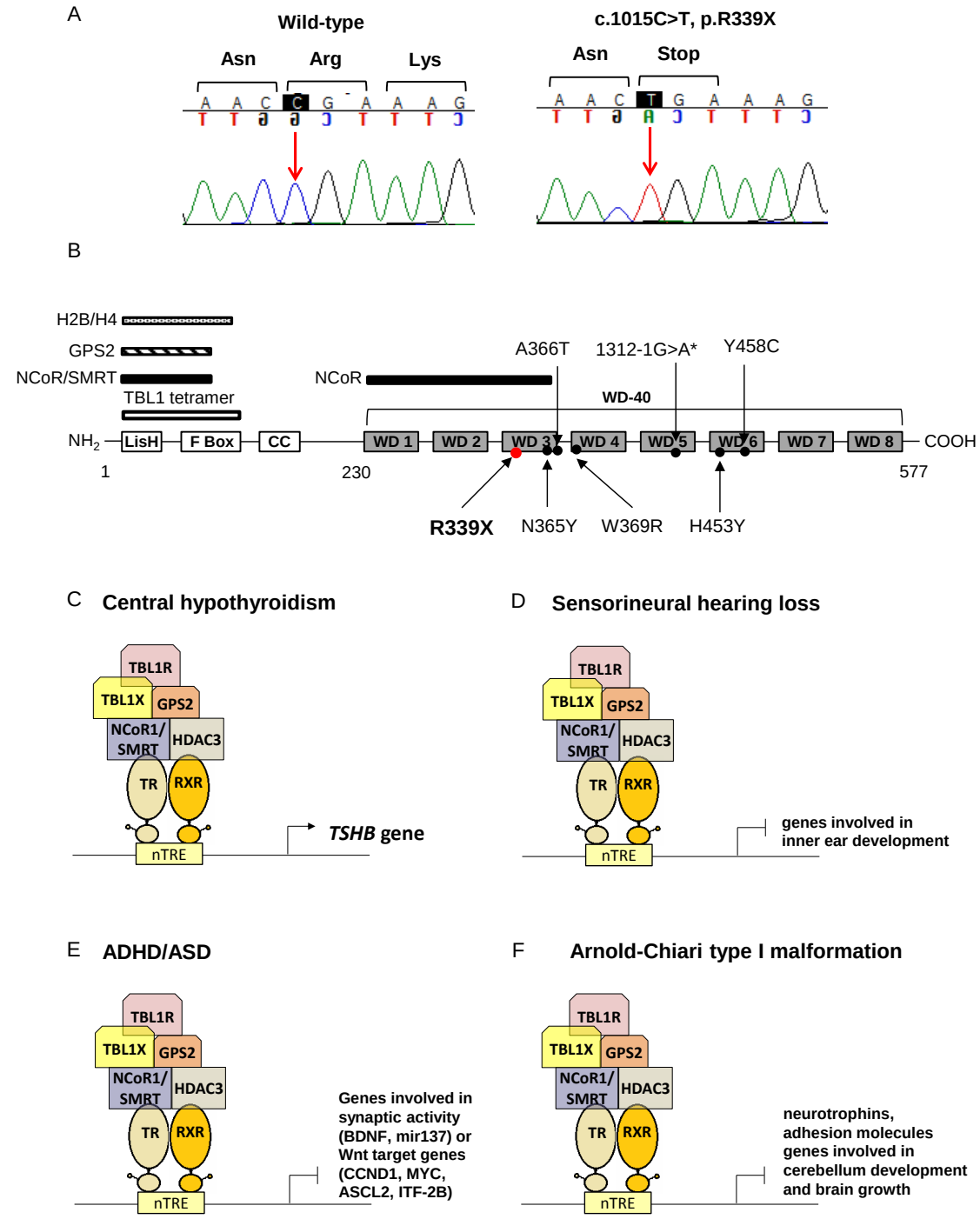
F




*(Legend in following page)*

**Figure 1: Clinical and biochemical features of the patient with TBL1X defect.** (A) Pedigree of members of the family showing patient born of twin pregnancy from non-consanguineous parents. Thyroid hormone profile and glycosylated haemoglobin were used to evaluate hypothyroidism and diabetes, respectively, in all members of the family. y: years of age, wt: wild-type, N.R.: normal range. In bold are represented values above normal ranges. (B) Thyrotropin response of the index patient (6 years of age without L-T4 treatment) at the TRH test during 180 minutes. TSH and prolactin values are represented in the graph through time during the 3 hours of the test. Fold-increase of TSH was calculated by dividing the TSH value at each time (0, 15, 30, 60, 120 or 180 minutes) by the TSH level at the beginning of the test (0 minutes). FT4 increase 3 hours after TRH administration was calculated as reported (van Tijn DA *et al.*, 2008B), as an indirect measure of TSH bioactivity and was normal. %FT4 Incr: percentage of free T4 increase. min: minutes. In italics are represented values below normal ranges. (C) Graphical correlation between TSH and FT4 values plotted in chart for normal thyroid and pituitary homeostasis (adapted from Dietrich (Dietrich JW *et al.*, 2012)). (D) Thyroid ultrasound at 6 years and 9 months of age, representing three dimensional measures of each lobule, showed mild hypoplasia of the gland. (E) Brain MRI in the index case at 6 years and a half of age showing normal pituitary morphology and size (white arrow) but an Arnold-Chiari type I malformation which compresses the spinal cord (red arrows). (F) Hearing loss per frequency in the right and left ear of the index patient are represented in above and below panels, respectively.




*(Legend in following page)*

**Figure 2: Genetics findings: index patient carried *de novo* non-sense mutation in *TBL1X* gene.** (A) Representative chromatograms showing wild-type and R339X *TBL1X* mutation in hemizygous state. (B) Scheme showing the location of R339X at the 3$^{th}$ WD-repeat motif of the protein (red dot) and the other *TBL1X* mutations previously described. R339X is the most N-terminal mutation described, located in a region of interaction with NCoR, probably disrupting the assembly of NCoR-SMRT complex over the thyroid hormone receptor. LisH and F Box motifs, located in the N-termination of the protein, are regions of interaction with different proteins as GPS2, H2B/H4 and NCoR/SMRT belonging to the thyroid hormone repressor complex. Moreover, this region is also involved in homo-tetramerization of the protein. Schematic representations of *TBL1X* action within NCOR-SMRT complex regulating different T3-responsive gene expression.(C) *TBL1X* stimulates expression of TSHB by repression of thyroid hormone action. Thus, defects in *TBL1X* cause central hypothyroidism. (D) *TBL1X* down-regulates the expression of genes involve in inner ear development by the repression of T3 action. Thus, defects in *TBL1X* cause hearing loss (E) *TBL1X* down-regulates the expression of genes involved in synaptic activity,like *BDNF* (brain-derived neurotrophic factor) or Wnt target genes, associated with ADHD or ADS (F) *TBL1X* down-regulates the expression of genes involve in cerebellum development and brain growth through repression of T3 action. Thus, *TBL1X*defects cause Arnold-Chiari malformation.

# CAPÍTULO VI

## Hipotiroidismo Central e Hipermetabolismo de origen Hipotalámico

El eje tiroideo es un modulador importante del balance energético y el metabolismo de lípidos. El estado de hipertiroidismo se caracteriza por un aumento en el gasto energético con la consiguiente pérdida de peso, acompañado de una marcada hiperfagia. Generalmente, los efectos más estudiados de la hormona tiroidea sobre el metabolismo se dan a nivel periférico. No obstante, recientemente se ha descrito el papel de la hormona tiroidea en el hipotálamo, regulando el metabolismo de lípidos complejos que actúan como neurotransmisores del sistema nervioso simpático (SNS) en la estimulación de la grasa parda. Contrariamente a lo que se pensaba, la grasa parda es un tejido termogénico que también está presente en determinadas localizaciones corporales en adultos y cuya activación conduce a un aumento de gasto energético y una pérdida de peso.

El objetivo del presente estudio se centra en la investigación clínica y etiológica de hipotiroidismo asociado a un remarcado hipermetabolismo en un pedigrí familiar no consanguíneo.

El caso índice, nacido a término en percentiles 50 de peso y talla, presentó un estancamiento de peso y talla inferiores al percentil 3 asociados a una marcada hiperfagia y gasto calórico excesivo. Este hipermetabolismo severo se asoció a los 18 meses de edad con un hipotiroidismo que se identificó de origen hipotalámico mediante test de TRH. El paciente requirió alimentación adicional por sonda nasogástrica y finalmente por gastrostomía a los 23 meses de edad para conseguir una recuperación de peso y talla por encima del percentil 3. Ante la sospecha de un origen central del hipermetabolismo, la paciente se sometió a un test de propanolol seguido de una calorimetría indirecta. La inhibición del SNS por el propanolol consigue disminuir el gasto energético en reposo (GER) en un 20%, confirmando el origen central de la enfermedad. Además, el estudio de la activación de la grasa parda mediante termografía infrarroja de alta resolución muestra una sobreactivación basal de la grasa que no es capaz de estimularse en respuesta al frío o a la ingesta. La presencia tanto de hipotiroidismo como hipermetabolismo en otros miembros familiares apoyan un componente genético de esta enfermedad que tendrá que ser estudiado.

En conclusión se ha identificado por primera vez un hipotiroidismo hipotalámico asociado a un hipermetabolismo de origen central por sobreactivación basal de la grasa parda. Se sugiere un componente genético en este nuevo fenotipo que será estudiado en futuras investigaciones.

# Central hypothyroidism and hypermetabolism of hypothalamic origin

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**Abstract**

The thyroid axis is an important modulator of energy balance and lipid metabolism. The state of hyperthyroidism is characterized by an increase in energy expenditure with consequent weight loss, accompanied by marked hyperphagia. Generally, the most studied effects of the thyroid hormone on metabolism occur at the peripheral level. However, the role of thyroid hormone in the hypothalamus has been recently described, regulating the metabolism of complex lipids that act as neurotransmitters of the sympathetic nervous system (SNS) in the stimulation of brown fat. Contrary to belief, brown fat is a thermogenic tissue that is also present in certain body locations in adults and whose activation leads to increased energy expenditure and weight loss.

The objective of the present study was the clinical and etiological investigation of hypothyroidism associated with a remarked hypermetabolism in a non-consanguineous family pedigree.

The index case, born at term in the 50$^{th}$ percentile of weight and height, presented stagnation of weight and height below the 3$^{rd}$ percentile, associated with marked hyperphagia and excessive caloric expenditure. This severe hypermetabolism was associated at 18 months of age with hypothyroidism that was identified as hypothalamic origin by TRH test. The patient required additional feeding by nasogastric tube and finally by gastrostomy at 23 months of age to achieve weight and height recovery above the 3$^{th}$ percentile. On suspicion of a central origin of hypermetabolism, the patient underwent a Propanolol test followed by indirect calorimetry. The inhibition of SNS by propanolol decreases resting energy expenditure (REE) by 20%, confirming the central origin of the disease. In addition, the study of the activation of brown fat using high resolution infrared thermography shows a basal overactivation of fat that is not capable of being stimulated in response to cold or food intake. The presence of both hypothyroidism and hypermetabolism in other family members supports a genetic component of this disease that will have to be studied.

In conclusion, hypothalamic hypothyroidism associated with hypermetabolism of central origin by basal overactivation of brown fat has been identified for the first time. We suggest a genetic component in this new phenotype that will be studied in future research.

## Introduction

Hypermetabolism is an excess of body energy expenditure that leads to deterioration in the nutritional status of the individual, even in hypercaloric dietary conditions. In humans, it is poorly described. However, a number of transgenic (TG) or gene deletions (KO) murine models involved in the energetic metabolism and their regulation at the hypothalamic level show clear hypermetabolism phenotypes with extreme thinness (Reitman ML, 2002; Sjögren M *et al*., 2007; Adams BA *et al*., 2008; Gelling RW *et al*., 2008; Yeh TY *et al*., 2009; Santiago LA *et al*., 2011; Sun C *et al*., 2012; Virtue S *et al*., 2012; Shumake J *et al*., 2001) (Table 1). In children, hypermetabolism would lead to severe malnutrition, lack of growth, and in adults to states of thinness that could be classified as "constitutional", perhaps with hereditary features. Both situations would constitute the "reverse mirror image" of obesity, a pandemic that seriously affects societies today.

Recently, the important role of the thyroid hormone (T3) in hypothalamus as a regulator of the afferent pathways in the sympathetic nervous system has been revealed, controlling the activity of brown fat, an essential tissue for energy expenditure for its thermogenesis capability (Figure 1A). Contrary to previously assumed, brown fat not only exists at very early ages in its interscapular location, but also recently shown its important thermogenic function in adults from alternative locations in the body (Enerbäck S, 2010). Basically, T3 hormone acting through its receptor TRα1 in the ventromedial nucleus of the hypothalamus, diminishes the activity of the AMPK, triggering the increase of hypothalamic lipid metabolism and the generation of complex lipids. These complex lipids act as a mode of neurotransmitters of the sympathetic nervous system (SNS), increasing the activity of brown fat, which increase thermogenesis and energy expenditure and reduce body weight (López M *et al*., 2010) (Figure 1A).

The essential role of AMPK enzyme in regulating energy homeostasis has been consistently demonstrated (Lage R *et al*., 2008; Blanco Martínez de Morentin P *et al*., 2011). The most novel is its tight negative regulation through the thyroid hormone and its receptor in the hypothalamus. It is also novel and requires a paradigm shift, that the metabolic effects of T3 classically known, as weight loss in patients with hyperthyroidism, are not solely due to the peripheral effects of the hormone on crucial genes in thermogenesis, like the mitochondrial UCP1 and UCP3 (Solanes G *et al*., 2005), it are also due, or perhaps mainly, to the central effects of T3.

As both the hypothalamic TRH gene and the 3 subunits of the enzyme AMPK (PRKAA1/2, PRKAB1/2 and PRKAG1/2/3) are transcriptionally regulated by thyroid hormone receptors (TRs), the association of a hypothalamic hypothyroidism and hypermetabolism of hypothalamic origin is plausible (Figure 1B).

## Clinical case

The index case is a girl of non-consanguineous parents, born to term in 50$^{th}$ percentile of weight and height (Figure 2A and B). At 9 months of age, she presented a stagnation of weight and height below the 3$^{rd}$ percentile, associated with marked hyperphagia and excessive caloric expenditure (BEE = 398 Kcal, > 1.5 times of normal for his age) (Table 2), along with a profuse night sweats. Therefore, at 18 months, the caloric intake was liberalized up to 3 times the normal consumption for its age, being observed an improvement in its weight and height (Figure 2B and Table 2).

The study of hormone levels at 18 months revealed a central hypothyroidism that had not been detected until then and began to be treated. The TRH test determined that the origin of central hypothyroidism was hypothalamic, with an excessive TSH response (high capacity and potency) incapable to recover its basal levels after 3h of TRH stimulus (Figure 2C) (Chapter I of this Thesis). Growth hormone (GH) and prolactin were within the normal ranges. Leptin, a marker of the amount of peripheral fat, was decreased, and the neuropeptide Y, a marker of appetite, was increased.

In view of the serious nutritional situation, additional feeding by nasogastric tube (NG) is indicated at 20 months (Figure 2B and Table 2), with a positive impact in both curves but without reaching the percentile 3, a milestone that is only achieved after implantation of nutrition through gastrostomy (Figure 2B and Table 2). It should be noted that the patient has only reached the 3$^{rd}$ percentile of weight and height at the expense of extreme caloric consumption that could damage her liver function (she has developed fatty liver) and probably renal function in the future (Table 2).

Given the hypothalamic symptoms of the patient (hypothalamic tumor suspected and discarded by magnetic resonance imaging) and the recent publications that associate a direct effect of hypothalamic T3 on the control of brown fat function through SNS (López M *et al*., 2010), we believe that there is a direct relationship between the hypothalamic thyroid hormonal alteration and the nutritional/metabolic alteration of this patient. To verify this hypothesis, the patient underwent an indirect calorimetry in the context of a propanolol test,

an adrenergic antagonist of SNS, to assess the origin of hypermetabolism. Indirect calorimetry performed 2 hours after propanolol administration showed a significant reduction in resting energy expenditure (REE) of 20% with respect to the indirect calorimetry performed before administration of propanolol (Figure 3).

These results strongly support that the increase in caloric expenditure presented in this patient is of central origin (hypothalamic) and not peripheral (of brown fat): after blocking (partially) the SNS signal on brown fat through propanolol, decreases its thermogenic activity.

In view of the suspicion of a central defect involved in the regulation of brown fat metabolism, the activity of this tissue was studied in the family pedigree using the technique of infrared thermography of high resolution. This noninvasive method determines brown fat activity in response to cold and food intake compared to healthy individuals of the same age and sex. The index case presented a basal surface temperature in brown adipose tissue higher than the mean of its age and sex (Figure 4A). However, this tissue was not activated either by cold or by ingestion (Figure 4B, C). The healthy controls of their age showed an increase of brown fat activation in response to the cold, reaching the basal values of the index case (Figure 4B). These results are compatible with a basal overactivation of brown fat in the index case and therefore not able to be further stimulated in response to cold or intake. The sibling of the index case (Figure 2A, III.2) had basal and stimulated normal brown fat temperature, comparable to healthy controls for sex and age (Figure 4A-C). The mother, although presenting a normal basal temperature, showed stimulus in response to the intake but not in response to cold. The father showed opposite results, with response to cold but not to intake (Figure 4A-C). In addition, it has been observed in healthy controls that there is an inverse correlation between basal surface temperature and BMI (data not shown).

Therefore, these findings support a common central hypothalamic origin for hypothyroidism and hypermetabolism, likely mediated by thyroid hormone. In this way, hypothalamic defects would be produce an over stimulation of the sympathetic nervous system, as has been observed by propanolol test, which leads to an increase in the basal metabolism of brown fat, identified by high resolution thermography.

In addition, the detailed study of the family pedigree supports a hereditary component of this phenotype: there are relatives affected by hypothyroidism in the two branches and alterations of satiety/excessive appetite in adult members of this family, who will be the first to be genetically investigated for this condition (Figure 2A).

| TH | Animal model | Gene | Phenotype | R |
|---|---|---|---|---|
| CENTRAL | KI mice | *Trα1* | Hypermetabolism, ↓ body mass, ↓ fatty deposits, hyperphagia, ↓ leptin, ↑ lipid mobilization, blockaded SNS that stimulates brown fat | 1 |
| | KO mice | *Mark4* | Hypermetabolism, ↓ fatty deposits, hyperphagia, resistance to obesity induced by diet, brown fat stimulation, insulin hypersensitivity | 2 |
| | KO mice | *Acc* | Hypermetabolism, hyperphagia, ↓ leptin, ↓ glucose, ↑ triglycerides | 3 |
| | KI mice | *Trβ* | ↓ Body mass, ↓ fatty deposits, hyperphagia, ↑ leptin, normal blood glucose, ↑ insulin sensitivity, ↓ insulin, ↓ gluconeogenesis | 4 |
| | KO mice | *Pacap* | ↓ Body mass, ↓ fatty deposits, ↑ insulin sensitivity, ↓ TRH, ↓ Dio2 | 5 |
| | Helpless rat | - | Hypermetabolism in the hypothalamus, ↓ body mass, hypophagia, ↓ physical activity | 6 |
| PERIFERIC | TG mice | *Ucp1* | Hypermetabolism, hyperphagia, ↑ insulin sensitivity, ↓ insulin, ↓ glucose, ↓ triglycerides | 3 |
| | TG mice | *Ucp3* | Hypermetabolism, hyperphagia, ↓ insulin, ↓ glucose | 3 |
| | KO mice | *Ptp1b* | Hypermetabolism, hyperphagia, ↓ leptin, ↑ insulin sensitivity, ↓ insulin, ↓ glucose, ↓ triglycerides | 3 |
| | KO mice | *Tnks* | Hypermetabolism, ↓ body mass, ↓ fatty deposits, hyperphagia, ↓ leptin, ↑ oxidation of fatty acids, ↑ use of glucose induced by insulin | 7 |
| | KO mice | *Adam17* | Hypermetabolism, ↓ body mass, ↓ fatty deposits, ↓ leptin, ↑ UCP1 | 8 |
| | KO mice | *L-Pgds* | ↑ Thermogenic genes expression | 9 |

***Table 1: Animal models for hypermetabolism***. *TH: Type of hypermetabolism, KI: Knock-in, KO: Knock-out, TG: transgenic, TRα1: Thyroid Hormone Receptor alpha 1, MARK4: Microtubule Affinity-Regulating Kinase, ACC: Acetyl-CoA Carboxylase 2, TRβ Thyroid Hormone Receptor beta, PACAP: Pituitary Adenylate Cyclase-Activating Polypeptide, UCP1/3: uncoupling protein 1/3, PTP1B: Protein Tyrosine Phosphatase 1B, TNKS: Tankyrase, ADAM17: ADAM17: metallopeptidase domain 17, L-PGDS: Lipocalin-type Prostaglandin D synthase, PVN: paraventricular nucleus, R: References.1: Sjögren M et al., 2007, 2: Sun C et al., 2012, 3: Reitman ML, 2002, 4: Santiago LA et al., 2011, 5: Adams BA et al., 2008, 6:* Shumake J *et al*., 2001, 7: Yeh TY *et al*., 2009, 8: Gelling RW *et al*., 2008, 9: Virtue S *et al*., 2012.

| Age (months) | 9 | 9-14 | 14 -18 | 18 | 20 | 21-23 | 23 | 23-32 | 32 |
|---|---|---|---|---|---|---|---|---|---|
| **REE** | 690 | 700 | | | 1157 | | 2023 | | |
| **BEE** | 398 | 404 | | | 495 | | 595 | | |
| **% BEE** | **173 (>x1.5)** | **176** | | | **232 (>x2)** | | **340 (>x3)** | | |
| **Food intake *(Kcal /day)*** | 1900 | 2200 | 2650-3000 | 2650-3000 | 1800 (NG)<br>2500 (oral) | 2600 | 2600-3000 | 3200 | 4200 |
| **Therapeutic action** | 1º attendance | ↑ calories intake | ↑ calories intake | Hypothalamic CCH | NG + oral intake<br>no ↓ REE | NG +oral intake | stop NG<br>gastrostomy | oral intake<br>gastrostomy | ↑ calories intake |
| **Height/Weight** | low | not improve | improve | improve | improve under ↓fasting periods | favorable | not improve<br>↓lean mass | favorable | favorable |

**Table 2: Evolution of patient's hypermetabolism.** REE: Resting energy expenditure, BEE: Basal energy expenditure, % BEE: indicates the percentage of the estimated basal energy expenditure, CCH: Central Congenital Hypothyroidism, NG: nasogastric tube.

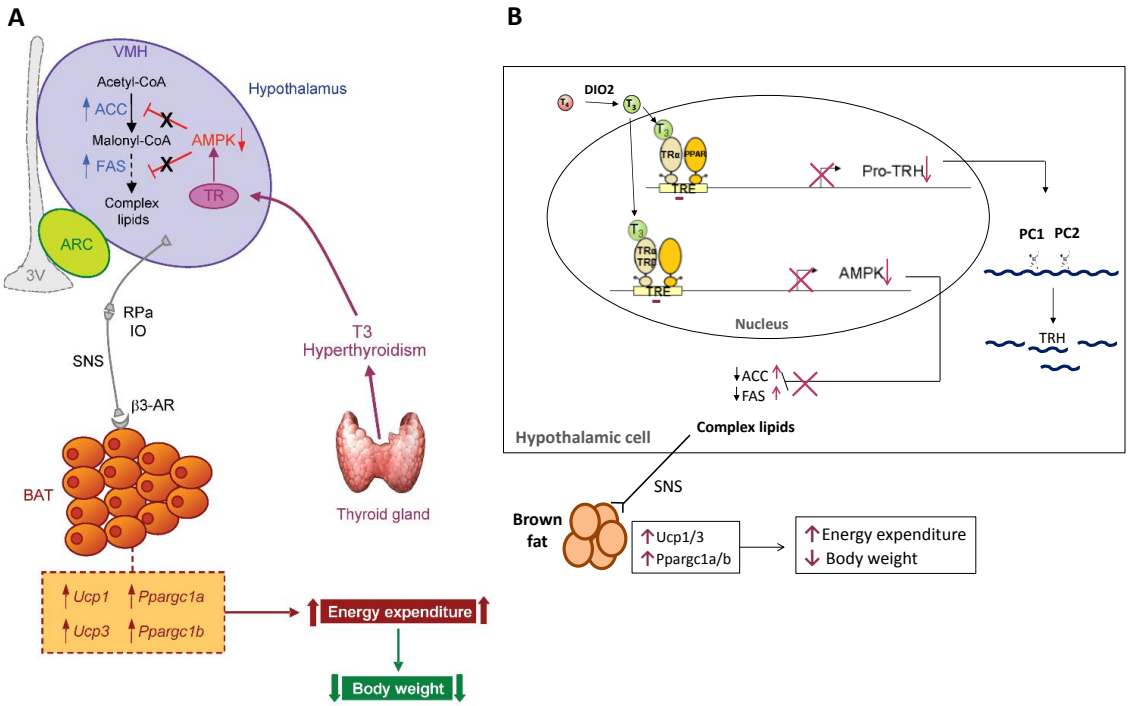


**Figure 1: (A) Model of the effects by excess hypothalamic thyroid hormones on fatty acid metabolism.** T3 positively regulates *de novo* lipogenesis in the hypothalamus, resulting from decreased AMPK activity, ACC activation and increased FAS expression. Thyroid hormone induces changes in the hypothalamus in *de novo* biosynthesis pathway of complex lipids by increasing malonyl-CoA levels. These changes are associated with the activation of the sympathetic nervous system (SNS) through the RPa (pale rafe nucleus) and the IO (lower olive nucleus), resulting, through β-adrenergic receptors, in a greater expression of brown fat markers (GPs) such as Ucp1, Ucp3, Ppargc1a (encoding PGC1α) and Ppargc1b (encoding PGC1β), leading to increased energy expenditure and weight loss. (ACA = acetyl CoA carboxylase, FAS = fatty acid synthetase, ARC = hypothalamic arcuate nucleus, SNS = sympathetic nervous system, T3 = triiodothyronine, PGC1α = peroxisome-proliferator-activated receptor-gamma co-activator 1α, UCP = uncoupling protein, AMPK = activated protein kinase). Adapted from M. López *et al*., 2010. (B) Thyroid hormone receptor (TR) as a hypothalamic transcriptional regulator. T3 negatively regulates the transcription of TRH and AMPK through their TRα and β receptors. The decrease in AMPK levels increases hypothalamic lipid metabolism, with the generation of complex lipids. These lipids stimulate the SNS that leads to stimulation of brown fat activity, increasing energy expenditure and reducing body mass.

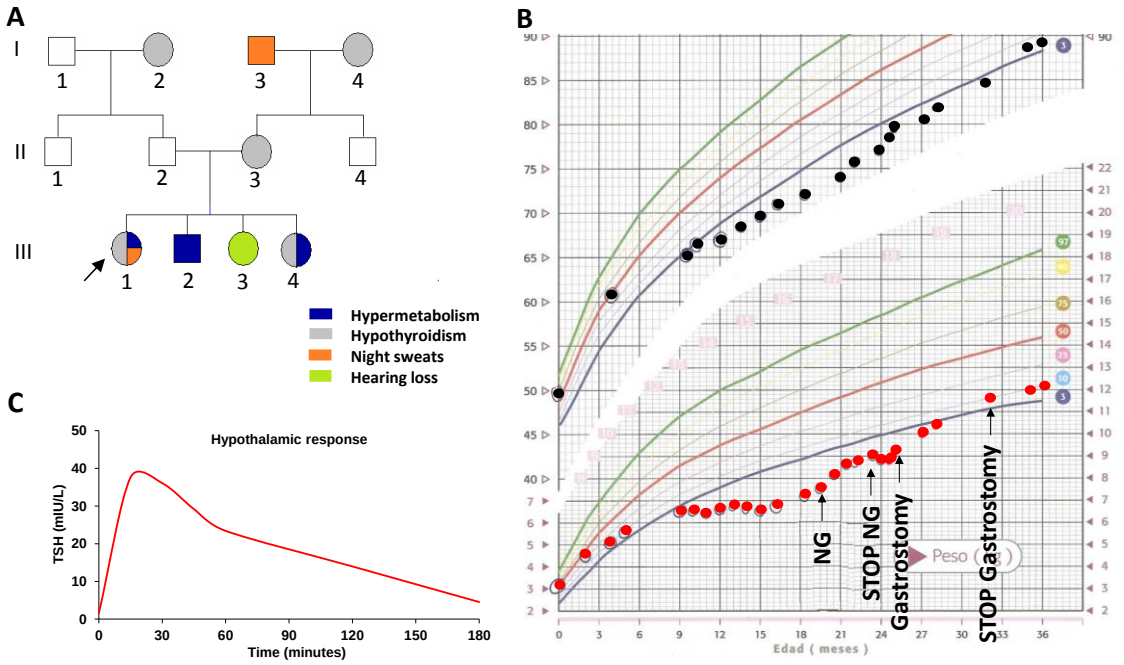


**Figure 2: Clinical follow-up of index case with hypothalamic hypermetabolism and hypothyroidism.** (A) Non-consanguineous family pedigree. Maternal and paternal history of hypothyroidism. Hypermetabolism of variable presentation in two of his brothers. (B) Evolution of weight and size of the index case over time, represented in growth curves for children (Carrascosa et al., 2008). The therapeutic actions are indicated in each point of the graph that corresponds to the weight of the patient at that age. (C) TRH test. Typical hypothalamic hypothyroidism profile with overstimulation response and absence of basal TSH recovery at 180 minutes.

| 10h of fasting | calorimetry | propanolol | 2 h | calorimetry | intake | 30 min | cal. | 30 min | cal. |
|---|---|---|---|---|---|---|---|---|---|
| % BEE | 180% | | | 160% | | 155% | | | |

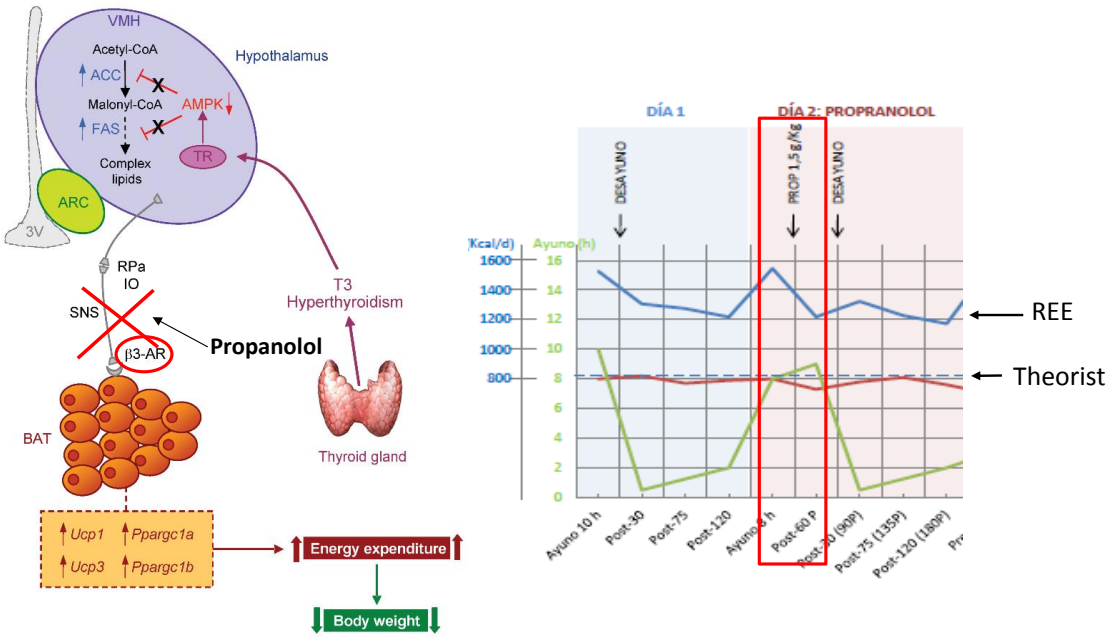


**Figure 3: Propanolol test.** Indirect calorimetry performed before and after the propanolol stimulus in order to determine resting energy expenditure (REE). Propanolol inhibits the sympathetic nervous system by blocking the beta adrenergic receptors, with a decrease in the REE of the patient after its administration, with respect to theoretical energy expenditure.

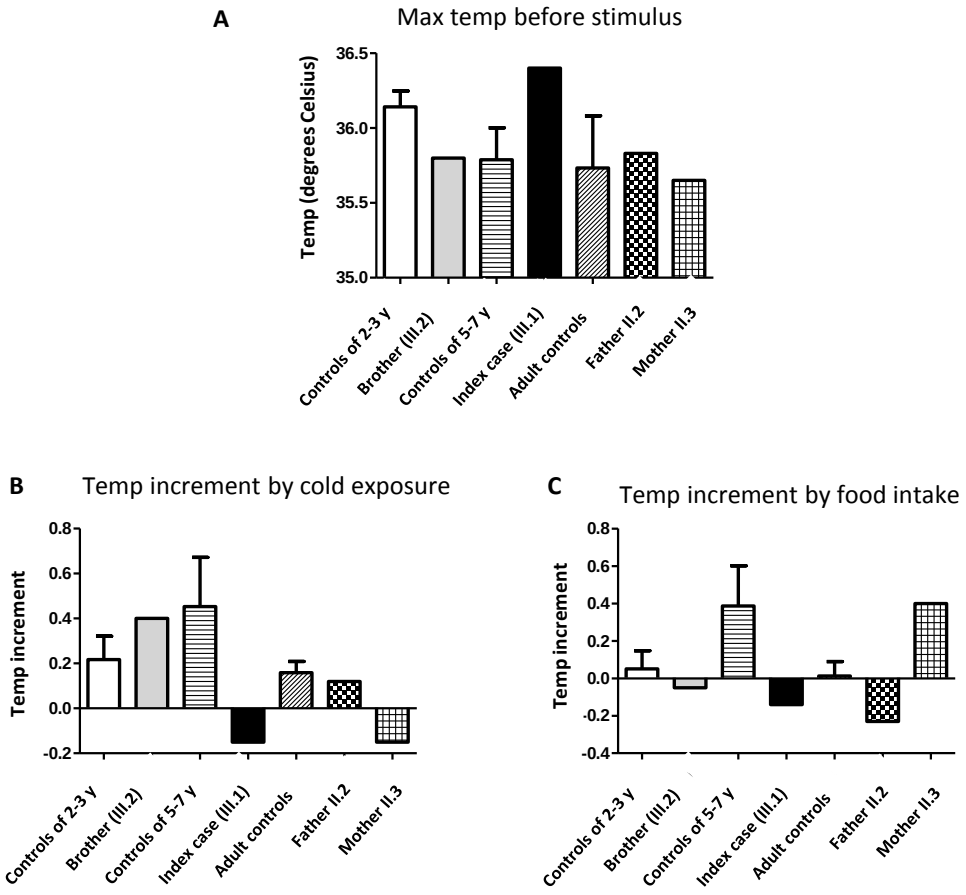


**Figure 4: High resolution infrared thermography**. (A) Body surface maximum temperature before stimulus. Index case showed higher surface temperature than her parents, brother and children and adult controls. (B) Temperature increment by cold exposure. Index case and her mother did not show temperature increment by cold stimulus, whereas her father and brother showed a similar increment than their respective controls. (C) Temperature increment by food intake. Index case, her father and brother did not show temperature increment by food intake, whereas her mother showed an exaggerated increment respect to adult controls. Thermography was performed in the index case at 6 years of age, using children aged 5 to 7 years as controls. In the brother it was performed at 3 years of age, therefore children between 2 and 3 years old were used as reference controls. Control adults were used for the parents. Temp: temperature, Max: maximum.

# DISCUSIÓN

*Marta García González. 2017.*

# *Hipotiroidismo congénito central: correlaciones clínico-genéticas e investigación de sus mecanismos moleculares*

# Discusión

En el presente estudio se ha investigado fenotípica y genéticamente la cohorte de pacientes con hipotiroidismo congénito central (HCC) más amplia conocida hasta la actualidad. Esta serie de pacientes es única por contener una gran proporción de casos con hipotiroidismo central aislado (68%). En el pasado, otros autores interesados en investigar el hipotiroidismo central lo hicieron con cohortes de pacientes que fundamentalmente presentaban defectos hormonales hipofisarios múltiples (van Tijn DA *et al.*, 2008B; Mehta A *et al.*, 2003), por ello el estudio fenotípico mediante test de TRH largo en nuestros pacientes con deficiencia de TSH aislada no tiene precedentes.

***Caracterización clínica***

En nuestra cohorte se investigaron pacientes diagnosticados tanto en edad pediátrica como adulta. Tan sólo estudiamos un paciente detectado en el cribado neonatal de Chipre, basado transitoriamente en la determinación de T4. En la mayoría de países europeos, incluyendo España, el cribado neonatal para hipotiroidismo sólo detecta aquellos de origen primario (tiroideo) por seguir la estrategia de determinación de TSH.

La sospecha de hipotiroidismo central a través de rasgos clínicos puede presentar dificultad, pues se manifiesta en ocasiones como signos y/o síntomas muy sutiles. Por tanto, el HCC en edad pediátrica suele pasar frecuentemente y en algunos casos permanece sin diagnosticar hasta la edad adulta. Algunas de las características diagnósticas de esta enfermedad en edad pediátrica incluyen talla baja, rendimiento escolar deficiente o actividad general letárgica (cansancio). La ausencia de retraso mental franco en muchos niños con HCC podría interpretarse como que su hipotiroidismo en el período neonatal y hasta los 3 años de vida haya podido ser leve. No obstante, no se debe subestimar la gravedad del hipotiroidismo neonatal central, ya que en los Países Bajos (donde utilizan un cribado neonatal basado en T4-TSH-TBG) un 55% de recién nacidos tienen hipotiroidismo severo o moderado (Zwaveling-Soonawala N *et al.*, 2015).

En nuestra cohorte, la edad media al diagnóstico de pacientes pediátricos con HCC está en torno a los 7 años. En el primer mes de vida sólo se diagnosticaron 6 niños. Estos datos sitúan el diagnóstico HCC neonatal en nuestra cohorte en un 14% (Capítulo I). En otras series de HCC, este porcentaje alcanza un 28% (Mehta A *et al.*, 2003), pero estas cohortes estudiadas incluían fundamentalmente niños con DCHP, que presentaron morbilidad neonatal derivada de otras deficiencias hipofisarias y que constituyen síntomas de alarma que llevan al diagnóstico más

precoz del HCC (van Tijn DA *et al.*, 2008B). Estos datos subrayan la dificultad de la detección de HCC *aislado* en países que no se benefician de programas de cribado basados en la determinación de T4. Por ello, en ocasiones estos pacientes se diagnostican en períodos de la edad infantil o adulta con requerimientos hormonales mayores, como la pubertad o en el embarazo en la mujer. En nuestra cohorte, una mujer fue diagnosticada ante dificultades para quedar embarazada. La falta de diagnóstico y tratamiento durante el embarazo en estas pacientes supone un riesgo para el neurodesarrollo para su futura descendencia. No sólo porque las mujeres pueden presentar hipotiroxinemia gestacional, lo que limita la cantidad de hormona tiroidea suministrada al feto via transplacentaria, fundamental para su desarrollo neurológico, sino también porque la propia capacidad de síntesis fetal de T4 (a partir de mediados el 2º trimestre del embarazo) pueda verse también reducido por transmisión del defecto genético al feto (Pine-Twaddell E *et al.*, 2013).

No obstante, en ocasiones pueden presentar signos fenotípicos que ayuden al diagnóstico de esta patología, tanto en HCC en el contexto de una DCHP (hipoglucemias, episodios de dificultad respiratoria, micropene) como en HCC aislado. Estos signos incluso pueden orientar el estudio del defecto genético, como es el caso de la presencia de macroorquidismo asociado al hipotiroidismo congénito central en defectos en *IGSF1* (Capítulo II). El diagnóstico de macroorquidismo es difícil a los 3 años de edad, sin embargo, el eje gonadal puede estar sobreestimulado desde el nacimiento en este trastorno, siendo detectado por el aumento de FSH durante la minipubertad neonatal o en un test de estímulo con GnRH en edad infantil (Capítulo II). Otro rasgo fenotípico asociado a hipotiroidismo central es la alteración en el volumen craneal, que se ha identificado en el transcurso de este estudio en cinco pacientes (8%) con macrocefalia (circunferencia de la cabeza para edad y género > 3 DS), macrocefalia relativa (tamaño de la cabeza en rango normal pero desproporcionadamente más grande en relación a la altura y peso del niño) o microcefalia (circunferencia de la cabeza para edad y género < 3 DS), respectivamente. Curiosamente, la investigación del origen genético del HCC en este grupo de pacientes identificó variantes en genes que forman parte del complejo co-represor de silenciamiento de los efectos de la hormona tiroidea (NCoR-SMRT), que modulan la expresión de TSHB en la hipófisis, como es el caso de *TBL1X, NCOR1 y GPS2* (Capítulo I y Capítulo V). Se necesitan estudios funcionales futuros para determinar la patogenicidad de las variantes identificadas y justificar que las alteraciones en el crecimiento craneal en estos pacientes sean debidas una vía de señalización común mediada por el complejo NCoR-SMRT. Esta vía está activa durante el desarrollo cerebro-craneal y posiblemente a través de la vía de señalización transcripcional Wnt-B-catenina (Capítulo V).

Finalmente, un 24% de niños con HCC aislado y un 4% con DCHP asociaron alteraciones del neurodesarrollo como déficit de atención, hiperactividad, dificultad en el aprendizaje, espectro autista o retraso psicomotor. Hasta la fecha no hay estudios que comparen el desarrollo psicomotor de los recién nacidos con HCC diagnosticados y tratados tempranamente (mediante cribado basado en T4) con el de los niños detectados a edades más tardías. Sin embargo, se han descrito niños diagnosticados después de los 3 meses de edad que presentaron retrasos psicomotores e intelectuales cuando se evaluaron a los 2-8 años (Bonomi M *et al.*, 2001; Dacou-Voutetakis C *et al.*, 1990; Baquedano MS *et al.*, 2010), lo que podría sugerir beneficios significativos derivados de una detección y tratamiento tempranos mediante el cribado con determinación de T4. Clásicamente, las características neurológicas de los niños hipotiroideos diagnosticados tardíamente se atribuyen a que la deficiencia de T4 afecta a la correcta maduración del cerebro. Aunque esta suposición tiene bases científicas sólidas a partir de evidencias clínicas y experimentales, proponemos que, en algunos casos de HCC, los defectos neurológicos podrían también derivarse de trastornos genéticos específicos, como es el caso del trastorno del espectro autista en los pacientes con defectos en *TBL1X* (Capítulo V).

***Dinámica de secreción de TSH en el test de TRH***

El test de TRH ha venido utilizándose en la discriminación de hipotiroidismo central de origen hipofisario o hipotalámico (Faglia G, 1998). No obstante, este uso no ha estado exento de controversia, ya que algunos autores evidenciaron respuestas hipotalámicas al test en hipotiroidismos atribuidos en exclusiva a defectos hipofisarios por evidentes malformaciones hipofisarias en RMN en el contexto clínico de DCHP (Mehta A *et al.*, 2003). Sin embargo, en Holanda van Tijn *et al* aplicó el test de TRH como complemento al cribado neonatal basado en T4, demostrando su utilidad en el diagnóstico etiológico certero del HCC (van Tijn DA *et al.*, 2008B). En estos dos estudios se realizaron protocolos diferentes del test de TRH. Mientras que Mehta y cols. llevaron a cabo un test corto, en el que se valoró como parámetros determinantes la capacidad máxima de secreción (pico) y el momento temporal del pico (Mehta A *et al.*, 2003), van Tijn y cols. utilizan un test largo de 3 horas de duración donde se evaluó tanto la capacidad de la hipófisis para sintetizar TSH cómo la dinámica de su secreción, tanto en la curva de subida hacia el pico de TSH como en su parte descendente o de recuperación de los niveles basales de TSH hasta el final del test (van Tijn DA *et al.*, 2008B).

En nuestro estudio, siguiendo básicamente el análisis van Tijn *et al*, evaluamos cuatro parámetros: como primer paso en la discriminación de los tipos de curvas de TSH se evaluó la

*capacidad total* de la hipófisis para la secreción de TSH (definida por el área bajo la curva, AUC), la capacidad máxima absoluta (definida por el pico de TSH) , y la capacidad máxima relativa o *potencia* (ratio pico de TSH/TSH basal) y la dinámica de secreción de la TSH a lo largo de la prueba, evaluando tanto el tiempo que tarda la TSH en alcanzar su máximo como su recuperación o no a niveles basales tras 3 horas de estímulo (Capítulo I).

Siguiendo estos parámetros, pudimos distinguir los 3 tipos básicos de respuestas de TSH a TRH: las respuestas normales (tipo 0 de van Tijn), pituitaria (tipo 2) e hipotalámica (tipo 3), pero también pudimos discriminar 2 subtipos diferentes de respuestas de predominio básicamente hipofisario (P1 y P2) e hipotalámico (H1 y H2), respectivamente (Capítulo I).

**La *respuesta tipo P1*** solapa con la respuesta hipofisaria clásica de van Tijn. Estas curvas tienen una capacidad total (AUC) y una potencia de pico de TSH muy disminuidas, pero con una dinámica de incremento y descenso de la TSH completamente normales, con recuperación final completa de los mismos niveles basales de TSH que existían al inicio del test.

***Las respuestas tipo P2*** presentan una capacidad total (AUC) también disminuida, pero la capacidad máxima relativa (ratio TSH pico/TSH 0´) está elevada, esto está en probable relación con el segundo rasgo característico de este tipo de curva: la ausencia de recuperación de TSH basales tras 180 minutos de estimulación.

| **Tipo de respuesta** | **Cap. total** | **Cap. Máx. Relativa** | **Tiempo pico** | **Recuperación de TSH** |
|---|---|---|---|---|
| | *(AUC)* | (*TSH pico/basal*) | *(15 ó 30 min)* | *(TSH 180'/0' < 1.5)* |
| P1 | ↓↓ | ↓ | N | Sí |
| P2 | ↓ | ↑ | N | No |
| H1 | ↑↑ | ↑ | N | No |
| H2 | ↑ | N | Retrasado | No |
| N | N | N | N | Sí |

**Tabla 1: Clasificación de los tipos de respuesta observados en pacientes con HCC sometidos a test de TRH.** Para ello se usaron diferentes parámetros: Cap. Total: capacidad total, representada por el área bajo la curva de respuesta (AUC), Cap. Máx. Relativa: capacidad máxima relativa, representada por la potencia del estímulo de TSH o ratio pico de TSH/ TSH basal, Tiempo pico: el tiempo que tarda la TSH en alcanzar su valor máximo, Recuperación de TSH a niveles basales: también representada por el ratio del valor de TSH a 180'/ TSH basal a 0'. P1: respuesta hipofisaria tipo1, P2: respuesta hipofisaria tipo 2, H1: respuesta hipotalámica tipo 1, H2: respuesta hipotalámica tipo 2. N: normal.

***La respuesta H1*** se caracteriza por una capacidad total de secreción de TSH (AUC) y una potencia de pico de TSH exageradas, alcanzando TSH séricas máximas muy elevadas a cronología normal (15 o 30 minutos). No obstante, la dinámica de recuperación basal de TSH

es muy lenta y se produce una secreción de TSH mantenida que no es capaz de recuperar los niveles basales tras 3 horas de estímulo.

**La respuesta H2** es una variante de la respuesta hipotalámica H1 con capacidad total (AUC) elevada, potencia del pico normal y una dinámica de secreción de TSH muy lenta que se traduce en un pico marcadamente retrasado (a los 45, 60 y hasta 180 minutos), con una dinámica de recuperación lenta o en algunos casos prácticamente inexistente (pico de secreción máxima al final del test). Curiosamente, todos nuestros pacientes con respuesta tipo H2 tenían DCHP asociada a la tríada de malformaciones hipofisarias y del tallo, que es compatible con la respuesta hipotalámica de van Tijn (tipo 3) observada en los pacientes que también presentaban DCHP y alteraciones morfológicas en la hipófisis. Este tipo de respuesta H2 merece un análisis conceptual específico porque podría ser la causa de las controversias sobre la capacidad de discriminación certera entre fallos predominantemente hipofisariso e hipotalámicos. Mehta *et al* argumentaban la poca capacidad del test en la discriminación etiológica, preguntándose ¿Por qué habrían de tener una respuesta hipotalámica (fallo hipotalámico) los pacientes con DCHP y malformaciones evidentes en la hipófisis? ¿Por qué el test de TRH no es capaz de traducir en una (esperable) respuesta hipofisaria los trastornos tan severos y demostrables en la hipófisis?

Recordemos que solo nuestro estudio identifica formalmente la existencia de estos dos subtipos de respuesta hipotalámica de TSH: Mehta *et al* no pueden identificarla por realizar test de TRH corto (siendo muy importante el criterio de no recuperación de la TSH basal) y van Tijn *et al*, que sí es capaz de evidenciar algunas de estas curvas en sus pacientes asimila las H2 en un grupo único de "respuestas por fallo hipotalámico" junto con las respuestas H1 de nuestra clasificación.

La solución definitiva de este enigma o de la discordancia entre respuestas "esperables" y respuestas "encontradas" o factuales en el test de TRH de pacientes con DCHP puede venir de la mano de la embriología del desarrollo y de un hallazgo radiológico muy particular en uno de nuestros pacientes con respuesta H2.

Es más aceptado que los procesos de desarrollo del hipotálamo y la hipófisis están íntimamente relacionados y que la formación correcta de hipófisis anterior, neurohipofisis y tallo hipofisarios se debe a estímulos morfogénicos que provienen del hipotálamo (Mehta A *et al.*, 2003). Por tanto, no sería extraño que las malformaciones frecuentemente descritas como hipofisarias (formando el síndrome de la tríada) realmente correspondan a malformaciones de la unidad hipotálamo-hipofisaria en el desarrollo. Sin embargo, la existencia de posibles

malformaciones hipotalámicas, incluyendo la hipoplasia, no son frecuentes, probablemente por no existir mucha experiencia en la definición de los bordes que delimiten el área hipotalámica para permitir comparaciones de tamaños de una manera homogénea, en ausencia de referencias consensuadas sobre su tamaño en distintas edades y sexo de la edad pediátrica. Recientemente se ha descrito una metodología para el estudio comparativo del tamaño y volumen hipotalámicos por técnicas especiales de RMN (Follin C *et al.*, 2016), basándose en la definición de los bordes anatómicos del hipotálamo establecidos por Gabery *et al.* (Gabery S *et al.*, 2014). Un método modificado ha permitido el diagnóstico en la paciente #24 de hipoplasia hipotalámica e hipofisaria, con respecto a controles de su edad y sexo (Comunicación personal Dr. Juan Martínez Sanmillan, Radiodiagnóstico, Hospital Ramon y Cajal. Madrid). Este hallazgo sugiere que, como en este caso, otros pacientes con respuesta H2 puedan también tener malformaciones hipotalámicas (no diagosticadas) asociadas a malformaciones hipofisarias más evidentes a la RMN clásica que, conjuntamente, sean las responsables de la DCHP que todos nuestros pacientes con H2 presentan. Estudios de RMN detallada están en marcha en este sentido para testar si el tamaño hipotalámico pudiera estar afectado estos pacientes con respuesta H2. En caso de confirmarse, el enigma de la existencia de respuestas hipotalámicas en casos con malformaciones hipofisarias (y del tallo) pudiera verse esclarecido.

Por lo tanto, proponemos una nueva clasificación para aquellos pacientes que han sido clásicamente incluidos en el grupo hipotalámico, pero que tienen defectos hipofisarios estudiados por resonancia magnética y perfil hormonal. Nuestra respuesta tipo H2 sería en realidad una respuesta "mixta" hipotálamo-hipofisaria con suficiente capacidad funcional de las células tirotropas como para secretar TSH ante estimulo de TRH exógena, aunque con un patrón muy aberrante.

***Defectos genéticos en el Hipotiroidismo Congénito Central***

Se ha identificado la causa genética en un 14% de los pacientes de nuestra cohorte, en los que se han detectado mutaciones patogénicas, un 4% con variantes probablemente patogénicas y un 24% de pacientes con variantes de significado incierto (15 variantes en total), quedando el resto sin variantes identificadas. La mayoría de las mutaciones patogénicas que determinan la causa del hipotiroidismo se encuentran en los genes clásicos relacionados con deficiencia de TSH. De esta forma hemos identificado una mayoría de alteraciones en el gen *IGSF1* que recientemente apunta a ser el defecto más frecuente en hipotiroidismo central, con más de 35 mutaciones identificadas hasta la fecha (Joustra SD *et al.*, 2016A).

***En IGSF1***

En el presente trabajo se han identificado un total de 4 pacientes con defectos en *IGSF1*, dos de ellos por deleciones completas del gen y otros dos por un defecto tipo inserción-deleción (INDEL) complejo. En la bibliografía se han identificado más de 20 familias con defectos en *IGSF1* que producen un fenotipo común de hipotiroidismo central asociado a otras características fenotípicas variables entre los pacientes como déficits parciales en otras hormonas hipofisarias, pubertad retrasada, hipoplasia tiroidea y alteraciones neurológicas como déficit de atención e hiperactividad, cuyas causas son por el momento desconocidas. Nuestros pacientes con deleción completa tienen un fenotipo muy similar entre sí, ambos fueron diagnosticados en periodo neonatal, aunque no en el cribado basado en TSH, por complicaciones derivadas de su hipotiroidismo y con un macroorquidismo muy llamativo desde los tres años de edad, otra característica que no siempre se manifiesta en este síndrome ligado al cromosoma X (Tajima T *et al.*, 2013). Ninguno de nuestros pacientes presentó pubertad retrasada ni ningún otro déficit hormonal, como se han descrito (Joustra SD *et al.*, 2016A). Pero ambos mostraron una respuesta muy llamativa de su eje gonadal, en concreto de la FSH. Uno de ellos presentó una FSH elevada durante la minipubertad y ambos mostraron estimulación de FSH en el test de GnRH, que es anormal a una edad prepuberal (cuando el eje gonadotropo esta silente) pero con una respuesta nula de testosterona. Esto sugiere fuertemente que el macroorquidismo es de origen hipofisario por un exceso de FSH que sobreestimula sus receptores testiculares (Capítulo II).

En este sentido se llevaron a cabo estudios experimentales para determinar tanto la localización celular de *IGSF1* como su mecanismo molecular de actuación. De esta forma se identificó la expresión de IGSF1 tanto en células tirotropas como gonadotropas de la hipófisis y también en células de Leydig y células germinales del testículo. Además, se ha visto que *IGSF1* estimula la transcripción del receptor de la hormona liberadora de tirotropina (TRHR) a través de la modulación negativa de la vía de señalización TGFβ1-Smad, favoreciendo de forma indirecta la síntesis y biopotencia de la TSH en células tirotropas. Por el contrario, IGSF1 regula negativamente la vía de señalización Activina-Smad, lo que conduce a una reducción en la expresión de FSHB en células gonadotropas. El mecanismo de actuación de IGSF1 en la hipófisis podría explicar cómo defectos en este gen producen una menor expresión de TRHR y una estimulación anormalmente elevada de la síntesis de FSH, conduciendo al hipotiroidismo central y al macroorquidismo (Capítulo II).

Se identificaron dos hermanos con un INDEL complejo en IGSF1 asociado a un fenotipo atípico para este síndrome ligado al X. Ambos debutaron con un hipotiroidismo central con TSH normal, pero tras la suspensión del tratamiento para reevaluación manifestaron una hipertirotropinemia muy marcada asociada a una severa hipoplasia tiroidea. Es la primera vez que se describe una hipertirotropinemia en pacientes con defectos en *IGSF1*, cuyo perfil hormonal generalmente se caracteriza por una deficiencia a TSH (TSH inapropiadamente normal). En este estudio demostramos la expresión de *IGSF1* en tiroides, por lo que este factor podría jugar un papel importante en desarrollo de la glándula y explicar (al menos en parte) el hipodesarrollo glandular tiroideo en esta enfermedad, y no como disminución del efecto trófico de la TSH sobre el tiroides (Capítulo III).

***En TRHR***

La identificación de defectos en el gen *TRHR* es infrecuente, sólo se conocen tres familias con defectos en el receptor y todos ellos abolen casi por completo su función (Collu R *et al.*, 1997; Bonomi M *et al.*, 2009; Koulouri O *et al.*, 2016). Este es el primer caso en el que se ha identificado un defecto leve en el receptor en un paciente de 8 años de edad en el contexto de una familia consanguínea. Este retraso en el diagnóstico ya se ha observado en los pocos pacientes que hay descritos en la literatura con defectos en este gen. Sólo uno de ellos fue diagnosticado en periodo neonatal, mientras que los otros dos fueron diagnosticados a la edad de 9 y 11 años por talla baja, respectivamente (Collu R *et al.*, 1997; Bonomi M *et al.*, 2009). Nuestro paciente no presentó talla baja, pero sí un sobrepeso que derivó en obesidad. Una característica particular de este defecto leve no observado hasta ahora es la presencia de hipertirotropinemia en los portadores heterocigotos del pedigrí familiar. Esta manifestación probablemente sea debida a la conservación parcial de la función del receptor mutante, ya que se trata de una mutación de cambio de aminoácido que no abole por completo la funcionalidad del receptor, pero claramente disminuye su capacidad de transactivacion y su afinidad por el ligando TRH, debido a un efecto alostérico conocido en otros GPCRs. (Capítulo IV).

***En TBL1X***

Actualmente son pocos los casos descritos de hipotiroidismo central por defectos en *TBL1X* (Heinen CA *et al.*, 2016). Este gen de herencia ligada al X se ha asociado muy recientemente con hipotiroidismo central, pero sería plausible que la frecuencia de cambios hallados aumentase con el tiempo como ha ocurrido con los defectos en *IGSF1*. En nuestra cohorte se han identificado dos pacientes con defectos patogénicos en este gen. Uno de ellos con una

deleción completa del gen, la primera descrita asociada a hipotiroidismo central, en el contexto de una deleción en el cromosoma X que causa un síndrome polimalformativo con fallo de varios ejes hipofisarios, retraso mental y déficit de atención e hiperactividad y rasgos compulsivos. Otro de nuestros pacientes presenta una mutación que produce un codón de parada prematuro que causa rasgos fenotípicos comunes a los pacientes descritos en la literatura pero con otros signos adicionales. La mutación hallada en este paciente constituye el cambio más N-terminal identificado hasta el momento, y se encuentra en un dominio de interacción con NCoR1, proteína fundamental del complejo co-represor del receptor de hormona tiroidea (NCoR-SMRT) del que forma parte *TBL1X*. Al igual que los pacientes previamente descritos, nuestro paciente presenta un hipotiroidismo central leve y un déficit auditivo en frecuencias altas. Sin embargo, observamos tres nuevas características no descritas en otros pacientes: una malformación de Arnold-Chiari, un déficit de atención e hiperactividad y una macrocefalia relativa (Capítulo V).

Curiosamente, muchas de estas características asociadas se han identificado en pacientes con variantes en genes que forman parte del complejo represor de la acción de la hormona tiroidea sobre la expresión génica (NCoR-SMRT), del que es miembro *TBL1X*. De esta forma, pacientes con variantes en NCOR1 y GPS2 también presentaron déficit de atención, retraso psicomotor y del aprendizaje y lenguaje y defectos del volumen craneal (micro y macrocefalia, respectivamente) (Capítulo I).

Defectos en proteínas co-activadoras (NCOs) de la acción de la hormona tiroidea sobre la expresión de *TRH* y *TSHB* a través de su receptor, y de heterodímeros con el receptor de retinoides (RXR), originan una resistencia central a hormona tiroidea en modelos de ratón (Takeuchi Y *et al.*, 2002; Costa-e-Sousa RH *et al.*, 2012; Astapova I *et al.*, 2011). No se han descrito mutaciones en RXRG asociadas a hipotiroidismo central en humanos, pero se conocen los efectos del bexaroteno (ligando específico de RXRG) que causa supresión de la secreción de TSH (Sherman SI *et al.*, 1999). En este estudio se ha identificado una variante en el gen RXRG en una madre y una hija con hipotiroidismo central que, curiosamente, presenta hipoglucemias con sospecha de un hiperinsulinismo. RXRG está presente en células B pancreáticas donde aumenta el metabolismo de la glucosa por aumento de expresión de GLUT1 (*Miyazaki S et al., 2010*). Se precisan futuros estudios más amplios de segregación familiar y estudios funcionales para determinar la implicación de esta variante en la enfermedad.

***En factores de transcripción implicados en síntesis de TSHB***

El porcentaje de detección de mutaciones en genes asociados a DCHP es muy bajo en los pacientes de nuestra cohorte. Esto es debido a que muchos genes causantes de hipoplasia o insuficiencia funcional de la glándula hipofisaria son desconocidos, por lo que en la mayoría de pacientes con estas características no se identifica la causa genética (Davis SW *et al.*, 2010). Hemos identificados una mutación patogénica en una familia consanguínea en el gen *POU1F1*, factor de transcripción fundamental para la expresión de *TSHB*, que es de herencia recesiva, esta mutación está descrita como patogénica y es la causante de su deficiencia combinada a TSH, GH y PRL (Bircan I *et al.*, 2001).

Además, se han identificado nuevas variantes de significado incierto en dos factores de transcripción que no se habían asociado con hipotiroidismo central en humanos hasta ahora. El receptor nuclear huérfano NR4A1 que se expresa en tirotropas pero también en corticotropas y gonadotropas, y participa en la activación transcripcional de *TSHB* junto a POU1F1 y GATA2, constituyendo un regulador del eje hipotálamo-hipófisis-tiroides (Nakajima Y *et al.*, 2012). Se ha identificado un paciente con una variante en *NR4A1* que presentaba una deficiencia de TSH combinada con hipogonadismo hipogonadotropo que le causó subfertilidad. El mecanismo molecular de la enfermedad mediada por este factor es desconocido y se necesitan futuras investigaciones para desvelar las implicaciones de NR4A1 en el eje gonadotropo. Además, estudios de segregación familiar y estudio funcionales in vitro de la mutación podrían determinar la patogenicidad del cambio estudiado.

En dos pacientes se han identificado variantes en el factor de transcripción ZFHX3 (Atbf1) que está implicado en activación temprana de la expresión del factor de transcripción POU1F1, esencial en la síntesis hormonal de células tirotropas, gonadotropas y lactotropas. (Qi Y *et al*., 2008). No se conocen mutaciones en humanos en este factor pero modelos de ratones KO de *Atbf1* presentaron baja expresión de Pou1f1, Lhx3 y Prop1, ausencia de proteína de TSHB y disminución algo menor de GH. Los pacientes de la cohorte portadores de cambios en este gen presentaron una leve deficiencia de TSH, con valores anormalmente normales para valores ligeramente reducidos de T4.

En resumen, se ha estudiado fenotípica- y genéticamente la serie más amplia conocida de pacientes con hipotiroidismo central aislado. Esta patología es escurridiza desde el punto de vista diagnóstico y en este estudio se aportan dos nuevas respuestas de secreción de TSH que tienen estos pacientes en respuesta a TRH, lo que está llamado a tener gran impacto clínico en esta patología.

Se han revelado por primera vez los mecanismos moleculares hipofisarios que explican de forma unitaria el hipotiroidismo central y un macroorquidismo asociado por defectos en *IGSF1*, y la intervención de rutas de TGF-B y Activina.

También es novedosa la identificación de nuevos fenotipos en genes conocidos asociados a HCC, como las hipertirotropinemias halladas en heterocigotos con defecto leve en *TRHR* o los defectos neuro-comportamentales identificados en dos pacientes con defecto severo en *TBL1X*.

Del mismo modo, los hallazgos genéticos causales identificados en este trabajo están en primera línea de la etiología recientemente conocida del hipotiroidismo central. Además, las novedosas técnicas masivas de genotipado (NGS) han revelado nuevos genes candidatos relevantes de la enfermedad como NR4A1, ZFHX3, NCOR1, RXRG o GPS2, entre otros. Que aseguran la continuidad de la investigación genético-etiológica de esta enfermedad que pasa tan desapercibida.

# CONCLUSIONES

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## Conclusiones

1. El diagnóstico del hipotiroidismo central aislado en países que no realizan cribado neonatal de la enfermedad es tardío, pues depende en gran medida de la pericia clínica y del mantenimiento de una alta sospecha diagnóstica. El modelo de relación dinámica entre TSH/T4 de Dietrich puede coadyuvar a esta sospecha clínica, y sustanciar la indicación de estudios etiológicos (test largo de TRH) y genéticos dirigidos (*Capítulo I de esta Tesis*).

2. El análisis detallado del test de TRH de 180 minutos es útil para el estudio etiológico del hipotiroidismo central, permitiendo discriminar el origen hipofisario e hipotalámico (o mixto), como lo indica la genética subyacente en el primer estudio que ha abordado la correspondencia fenotipo-genética de esta enfermedad. Algunos hipotiroidismos centrales (comprobados genética y funcionalmente) pueden tener un perfil de secreción normal de TSH en el test de TRH (*Capítulo I de esta Tesis*).

3. La clasificación específica del hipotiroidismo central en distintos subtipos sirve de base para la investigación fenotipo-genotípica que ha de caracterizar la nueva etapa de conocimiento molecular de la enfermedad que abre la secuenciación masiva. El análisis de tres parámetros en la secreción de TSH: capacidad total (AUC), potencia (ratio TSH pico/ basal) y dinámica (temporalidad del pico y recuperación o no de la TSH basal al final del test –ratio TSH 180´/0´-) sugieren la existencia de 5 tipos de respuesta al test: 2 con fallo primordialmente *hipofisario* (P1 y P2), 2 de fallo predominantemente *hipotalámico* (H1 y H2) y respuestas normales (N) (*Capítulo I*).

4. La clasificación del perfil secretorio de TSH propuesta halla justificación genética en *fallos hipofisarios P1* (defectos bialélicos o hemicigotos de *IGSF1, POU1F* y *TBLX1*) y en las respuestas N (mutación homocigota leve de *TRHR*) (Capítulo I). La *respuesta hipotalámica H1*, encuentra apoyo patogénico -aún no genético- en la identificación de un fallo del control hipotalámico de la actividad de la grasa parda en una niña con hipotiroidismo hipotalámico. Por último, la homogeneidad clínica y radiológica de los pacientes apoyan la existencia de la *respuesta hipotalámica H2* (todos con DCHP, y malformaciones hipotálamo-hipofisarias y síndrome de la tríada) (*Capítulo I*).

5. Nuestro abordaje genético del HCC (panel dirigido de NGS) identificó defectos en el 26% de pacientes de la cohorte, en genes conocidos como causantes de hipotiroidismo central, bien aislado (*IGSF1*, *TRHR*, *TBL1X*), bien dentro de DCHP (*POU1F1)*. También se identificaron variantes de perfil muy patogénico en genes que demostradamente regulan la síntesis de TSH pero aún no se han asociado con el HCC humano*: NR4A1, ZFHX3, RXRG, NCOR1, NCOA1, NCOA3 y GPS2*. En este último grupo de hallazgos se necesitan estudios de co-segregación familiar y análisis funcionales concluyentes para elevar estos genes candidatos a la categoría de genes causantes de hipotiroidismo central (*Capítulo I*).

6. Se han identificado dos mecanismos moleculares relevantes que explican las principales manifestaciones del "Síndrome de Hipotiroidismo Central y Macroorquidismo ligado al cromosoma X" por defectos en el gen *IGSF1*. Los defectos

en este gen producen una menor expresión del *TRHR* en células tirotropas y a la vez una sobre-expresión en la síntesis de *FSHB* en gonadotropas. Estos cambios conducen respectivamente a un fallo de síntesis de TSH (que además tiene bioactividad reducida) e hipotiroidismo, y paralelamente una excesiva secreción de FSH (evidenciable ya en la mini-pubertad neonatal) que, por hiperestimulación crónica del testículo (FSHR en las células d Sertoli) produce el macroorquidismo infantil. Las vías de señalización celular implicadas en estos dos fenotipos son la modulación negativa de IGSF1 sobre la ruta TGFβ y la modulación negativa de IGSF1 sobre la ruta de la Activina, respectivamente (*Capítulo II*).

Por otro lado, se ha determinado la expresión de *IGSF1* no solo en hipófisis, sino también, y por primera vez, en el propio tiroides humano. Proponemos que la actividad fallida de IGSF1 en esta localización del eje tiroideo pudiera estar implicada en la patogenia de una hipoplasia tiroidea (de intensidad variable) que se describe en pacientes con este tipo de hipotiroidismo central (*Capítulo III*).

7. Se han identificado fenotipos hormonales *desconocidos* en defectos de genes *conocidos* de hipotiroidismo central. Describimos por primera vez la existencia de hipertirotropinemia de origen central por mutación patogénica leve de *TRHR* que en homocigosis lleva a un hipotiroidismo central franco en una familia consanguínea. La mutación identificada provoca un cambio de polaridad en un motivo hidrofóbico implicado en los cambios conformacionales necesarios para la activación de la proteína Gsq, y por un mecanismo alostérico disminuye la afinidad del receptor por su ligando TRH (*Capítulo IV*).

8. Describimos por primera vez el fenotipo humano completo por defecto severo en *TBL1X*, que incluye HCC en el contexto de fenotipos malformativos y neuro-comportamentales hasta ahora no identificados como parte del síndrome: déficit de atención y rasgos autistas, encopresis, macrocefalia relativa y malformación Chiari I de la unión cráneo-vertebral (*Capítulo V*).

9. Por primera vez se ha identificado un HCC hipotalámico humano asociado a un hipermetabolismo energético de origen central (también hipotalámico) por sobre-estimulación funcional de la grasa parda periférica mediada por el sistema autonómico simpático (*Capítulo VI*).

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# ANEXO

*Marta García González. 2017.*

# *Hipotiroidismo congénito central: correlaciones clínico-genéticas e investigación de sus mecanismos moleculares*

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# Central Hypothyroidism in Children

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**Abstract**

Central congenital hypothyroidism (CCH) is an underdiagnosed disorder poorly described in childhood and adolescence. Congenital defects in thyroid-stimulating hormone (TSH) synthesis, secretion or bioactivity may lead to a state of 'regulatory' hypothyroidism expressed through aberrantly low or normal TSH levels and low thyroxine ($T_4$), a hormonal pattern undetectable by TSH-based neonatal screening programs for congenital hypothyroidism (CH) implemented in most countries worldwide. CCH is more prevalent than previously thought, reaching 1 in 16,000 neonates in countries consistently identifying CCH through $T_4$-based CH screening strategies. Neonatal detection and early treatment of CCH would prevent the risk of developing mental retardation secondary to late diagnosis of infantile hypothyroidism. CCH is frequently associated with other pituitary defects causing life-threatening situations (like e.g. adrenocorticotropic hormone deficiency) which could benefit from the early detection of CCH, avoiding considerable morbidity and mortality. CCH is not easy to identify clinically, and therefore few children are investigated for the disorder. The current knowledge on the genetic bases of CCH is also scarce. At the hypothalamic level no gene defects causing CCH have yet been identified in humans, but pituitary (thyrotrope)-selective genes encoding the TSH-releasing hormone (TRH) receptor *(TRHR)*, the TSH β-subunit *(TSHB)* and, recently, the immunoglobulin superfamily factor 1 *(IGSF1)* are genes involved in isolated central hypothyroidism. Moreover, central hypothyroidism is a complex condition where many regulatory signals are implicated and converge to finely modulate the activity of the hypothalamic-pituitary-thyroid axis. This review focuses on novel pathogenic mechanisms and their implications to understand human CCH and improve the identification and the therapeutic handling of this elusive disease in the pediatric age.



Central congenital hypothyroidism (CCH) is a heterogeneous group of diseases causing decreased synthesis, secretion or bioactivity of thyrotropin (thyroid-stimulating hormone, TSH), which fails to properly stimulate an otherwise normal thyroid gland. This condition is not detected by the widely implemented TSH-based neonatal screening programs for congenital hypothyroidism (CH) worldwide. For this reason CCH is an underdetected entity during the neonatal period, being mostly diagnosed during

infancy or childhood, if not in adulthood. CCH may manifest itself, but not always, as a clinically moderate hypothyroidism, with short stature as diagnostic hallmark, and allegedly lacking the consequence of psychomotor retardation. The molecular bases of CCH are largely unknown. Only defects in 3 genes have been identified as cause of CCH in humans: the classic *TSHB* and *TRHR* genes, encoding the β-subunit of TSH and the TSH-releasing hormone (TRH) receptor, respectively, but recently novel genes like the pituitary-expressed *IGSF1* (immunoglobulin superfamily factor 1) added to this short list responsible for a type of central hypothyroidism associating macroorchidism.

Given the physiological complexity of the central regulation of thyroid hormone synthesis, it is plausible that defects causing CCH may encompass a much broader genetic spectrum, involving more genes that to date remain unknown. Identifying further genes involved in CCH may be the key to a better understanding of the molecular pathophysiology of the disease, a goal demanding a higher degree of clinical suspicion, rate of diagnosis among pediatric endocrinology patients and the detailed characterization of the natural course of CCH through serial hormone determinations, morphological studies of the hypothalamic-pituitary area and dynamic TSH stimulation tests (TRH test) or in vitro TSH bioactivity assays.

## Neonatal Screening Programs for Central Congential Hypothyroidism

CCH is not detected by neonatal screening programs for CH followed in most countries, using the elevation of TSH as a marker reflecting typically primary (thyroidal) hypothyroidism, the most frequent type of CH.

When mass screening programs for CH and metabolic diseases were introduced in the early 80s of the last century, only few countries, including the Netherlands, Japan and some States in the USA, opted for thyroxine ($T_4$)-based screens aiming at the detection of all types of hypothyroidism. This has hampered estimations of the real prevalence of CCH until recently. Early global surveys in North America estimated the CCH prevalence to be 1 in 100,000 neonates [1] while such a prevalence was reported to be substantially higher (1:29,000) in the Northwest Regional screening program specifically using primary $T_4$ + TSH determinations [2]. In the Netherlands, under similar methodology, the CCH incidence was estimated to be 1 in 25,000 babies as for the initial 9 years of the Dutch screening (1981–1990). Such a program allowed the detection of some cases of CCH, but not all of them, as evidenced later [3]. In 1995, the Netherlands improved its national CH neonatal screening by adding the quantification of TBG (thyroxine-binding globulin) to the total $T_4$ and TSH concentrations from blood spot filter paper. This modification allowed calculation of the $T_4$/TBG ratio, used as substitute for free $T_4$ ($FT_4$), which is technically difficult to determine from filter paper eluates. $T_4$/TBG ratios properly discriminated the common TBG synthesis defects from real CCH cases. By introducing such a ratio, the effectiveness of CCH detection tripled (from 22 to 92%), indicating that the CCH prevalence could reach 1:16,000



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newborns [4]. A different optimization of the neonatal identification of CCH was recently developed in Japan. Using enzyme-immunometric assays in filter paper blood eluates, an accurate determination of $FT_4$ was achieved and implemented in the Kanagawa Prefecture CH screening program where the CCH prevalence was at least 1 in 31,000 infants [5]. The program suggests that this incidence probably represents a subestimation amenable to further refinement through additional technical improvements and detailed definition of best $FT_4$ thresholds to apply. Regarding cost-effectiveness of $T_4$-based programs, only 1 study is available, suggesting that the Dutch $T_4$-TSH-TBG screen is not substantially more expensive than the $T_4$-TSH protocol, when calculations are performed per CH patient detected [6]. No formal studies are yet available comparing psychomotor development of CCH infants with late diagnosis versus those early detected and treated through the mentioned screening programs. However, CCH children diagnosed after 3 months of age reveal psychomotor and intellectual delays when evaluated at 2–8 years [7–9] suggesting important benefits could derive from the neonatal detection of CCH. Aside the potential prevention of mental deficits, an additional benefit of programs detecting CCH is the early detection of concomitant pituitary failures in 78% of CCH cases, like e.g. adrenocorticotropic hormone deficiency, with high morbidity and mortality [10]. Such combined pituitary hormone deficiencies are otherwise diagnosed in early infancy with yet present dangerous symptoms [11].

Finally, early diagnosis and clinical follow-up of CCH would advance our knowledge of the natural behavior of infantile CH and put forward the identification of novel phenotypes of pituitary-hypothalamic disease, as exemplified by the recently unraveled *IGSF1* defects [12–15], mostly characterized in Dutch and Japanese patients. Genetic investigations in families from other early diagnosed CCH patients may well uncover previously unknown molecular mechanisms for this clinically elusive disease.

## Central Control of Thyroid Function: Hormonal and Molecular Actors

Thyroid hormones are essential for the correct development, differentiation and function of many cell systems of the organism, regulating cellular metabolism and being critical for brain development all through embryonic, fetal and postnatal life. Such important function anticipates tight regulation of the thyroid gland, exerted from central brain structures at the hypothalamus and the anterior pituitary.

Three main hormonal signals are involved in the control of the hypothalamus-pituitary-thyroid axis: TRH produced by neurons at the paraventricular nucleus (PVN) of the hypothalamus, TSH synthesized in specialized thyrotropes of the pituitary, which sequentially modulates the synthesis and secretion of $T_4$ and triiodothyronine ($T_3$) by the thyroid [16] (fig. 1a). In peripheral tissues, the system of iodothyronine deiodinases activates (DIO1, DIO2) or inactivates (DIO3) either iodothyronines to meet



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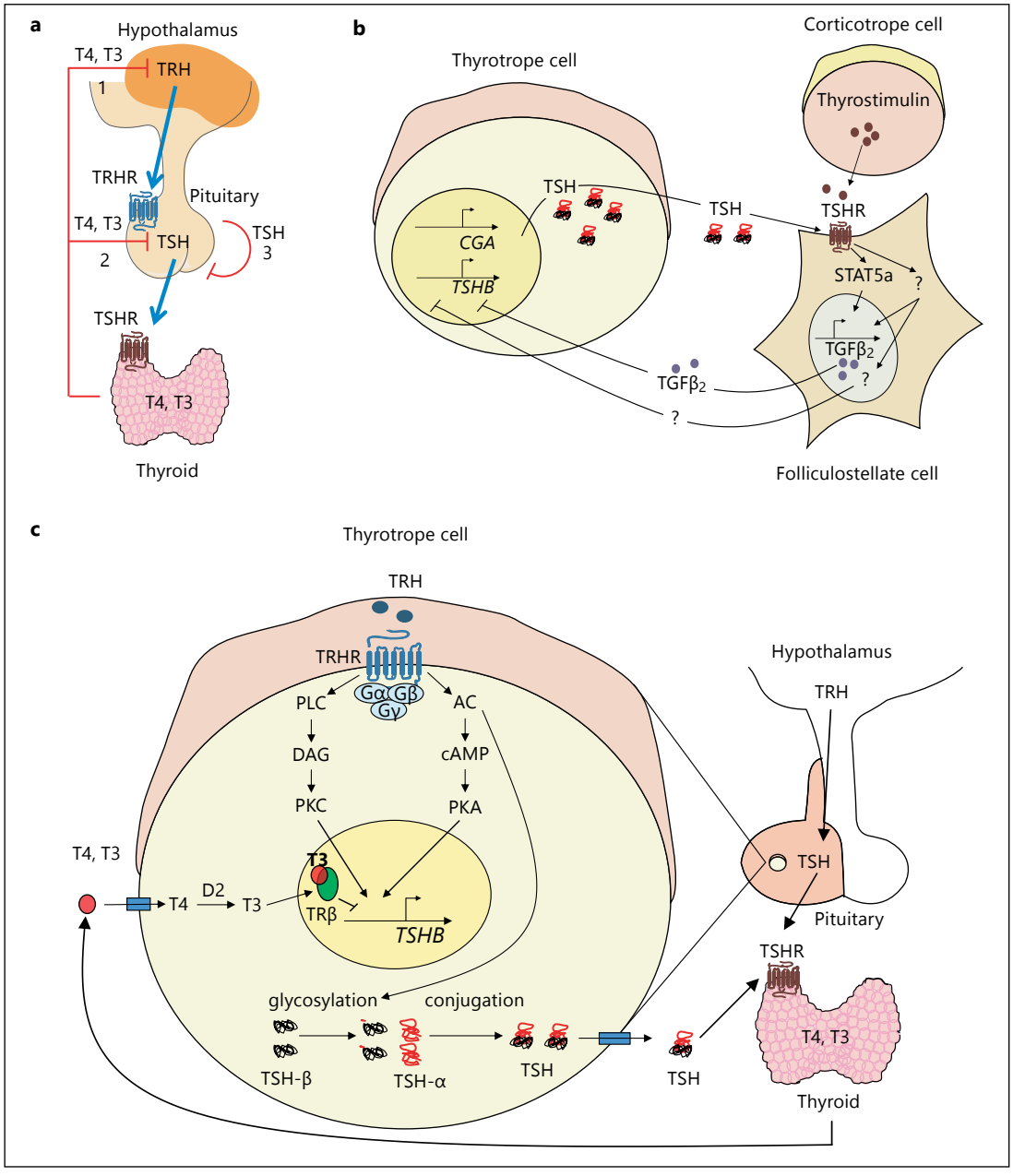


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individual needs of each particular tissue [17], and they actively participate in the negative feedback of the axis by the iodothyronines at central structures.

Therefore, the pituitary thyrotropes represent the crossroad where stimulatory and inhibitory signals of the thyroid axis converge and integrate into TSH outputs that can be modulated in magnitude and biopotency. In addition to this feedback hormonal system, hierarchically higher and thyroid hormone-independent pathways seem to be



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able to override $T_4$-$T_3$ regulation in response to physiological conditions such as fasting, low temperature and circadian rhythms orchestrated through neural circuitries [18]. All in all, thyroid hormone homeostasis is the result of a highly interactive system where dysregulations of centrally controlled mechanisms, summarized as follows, may well result in central hypothyroidism, from disconnected feedback loops or from feed-in autonomously generated signals that perturb a finely tuned equilibrium of centrally converging mechanisms [19].

## *Hormonal Mechanisms Controlling Thyroid-Stimulating Hormone Action*

### *Modulation of Stimulatory Signals for Thyroid-Stimulating Hormone Action*

#### TRH Synthesis and Degradation

TRH is a tripeptide (pyroGlu-His-Pro) produced in the PVN of the anterior hypothalamus. It is synthesized as a propeptide precursor of 242 amino acids and posttranslationally processed by endopeptidases (proconvertases 1 and 2) which release 5 mature TRH molecules per propeptide molecule [20]. Endopeptidases therefore represent a first regulatory step of the amount of mature TRH molecules with functional impact [21].

TRH molecules are secreted in the median eminence to reach and bind specific receptors (TRHRs) at the plasma membrane of the thyrotropic cell. Once it is secreted, TRH can be degraded by the enzyme pyroglutamyl peptidase II, suggesting an additional regulatory step of the amount of TRH molecules factually reaching the pituitary thyrotropes [22].

#### Pulsatility of TRH and TSH Secretion

Like other pituitary hormones, TSH is released in pulses, an intrinsic characteristic of its mode of action. TSH pulses have a frequency of 5–20 per day and show an average amplitude of 0.6 mU/l [23]. These pulses occur superimposed to a basal circadian rhythm that leads to a maximum TSH secretion at midnight, progressively decreasing until the evening on the next day [24].

**Fig. 1.** Hormonal and transcriptional regulation of the thyroid hormone axis. CGA = Glycoprotein hormones α-subunit; PLC = phospholipase C; DAG = diacylglycerol; PKC = protein kinase C; AC = adenylate cyclase; PKA = protein kinase A. **a** Negative feedback loops in the control of hormonal thyroid axis: (1) long loop thyroid-hypothalamus, (2) short loop thyroid-pituitary, (3) ultrashort loop within the pituitary. **b** Paracrine secretion of TSH in pituitary stimulates TSHR in folliculostellate cells, which stimulates the STAT5a pathway inducing secretion of transforming growth factor $\beta_2$ (TGF-$\beta_2$) or similar molecules that inhibit synthesis of TSHB at the thyrotrope cell. Thyrostimulin seems to have a paracrine regulatory role on this negative feedback also by stimulation of the TSH receptor in folliculostellate cells. **c** TSH synthesis, secretion and bioactivity signaling pathways activated by TRHR in thyrotrope cells. *TSHB* transcription is stimulated by two different signaling pathways (PLC and AC) activated by hypothalamic TRH. Additionally, the AC pathway promotes the correct glycosilation of TSH and its biopotency. Then, the β-subunit is conjugated with the α-subunit forming the active TSH dimer capable to stimulate the thyroid gland. Thyroid hormone receptor β is essential in the short negative feedback thyroid-pituitary repressing the *TSHB* synthesis.



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The intrinsic mechanism triggering the pulsatile secretion of TSH is largely unknown, probably having a major hypothalamic origin. TRH is also secreted in a pulsatile manner in the hypothalamus [25]; however, it seems that TRH only modulates the amplitude of TSH pulses, but not their frequency [26], suggesting a pituitary component is also active in the TSH pulse generator. Indeed, the pulsatile secretory profile of TSH is shown to be maintained in pituitaries experimentally 'disconnected' from the hypothalamus in animal models [27]. Loss of TSH pulsatility is seen in human diseases like the nonthyroidal illness or thyrotropinomas [28, 29]. Leptin has been described to have a role in the regulation of the circadian TSH rhythm [30]. In agreement with such a role, central hypothyroidism is present in leptin-deficient *ob/ob* mice, probably due to leptin stimulation of gene expression of pro-TRH and of proconvertases 1 and 2 [31].

Desensitization of Pituitary TRHR to TRH

In the pituitary, TRH stimulates its specific receptor TRHR at the cell membrane of thyrotropes. After TRHR activation, a fast desensitization of the receptor to the TRH ligand occurs through phosphorylation of Ser/Thr residues of the TRHR cytoplasmic tail by the G-protein-coupled receptor (GPCR) kinase 2. Subsequently, arrestins internalize the receptor by endocytosis, quickly preventing the possibility of prolonged stimulation of the receptor by TRH. Once TRH disappears from the thyrotropic cell milieu, phosphatase 1 starts to dephosphorylate the TRHR molecules at the endosomes allowing their transport back to the membrane for initiation of a new cycle [32].

TRH Control of TSH Biopotency

TRHR signaling induces not only *TSHB* transcription, but also governs the posttranslational modifications (mainly glycosylation) of TSH allowing its intrinsic bioactivity (fig. 1c). Different variants of TSH molecules exist depending on the type of carbohydrates added to asparagine 43 (N-glycosylation) and the final conformation of carbohydrate chains at this position. A high sialic acid content of TSH decreases its biological activity and increases its half-life [33]. By contrast, non-sialylated forms of TSH show increased biopotency and are captured by the asialoglycoprotein receptor in hepatocytes and rapidly degraded. Different forms of TSH glycosylation may therefore contribute to thyroid homeostasis and be implicated in disease: an increase in TSH sialylation would result in a lower bioactivity which may explain the lack of correlation between the levels of TSH and $FT_4$ in some patients with central hypothyroidism [34].

## *Modulation of Inhibitory Signals for Thyroid-Stimulating Hormone Action*

Thyroid hormones are the main executers of the downregulation of their own axis at the hypothalamus and pituitary, controlling TRH and TSH secretions, respectively.

This negative regulation of the axis takes place through 3 negative feedback 'loops' or circuits that occur centrally: a *long* loop (action of $T_3$ and $T_4$ at the hypothalamus),



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a *short* loop ($T_3$ and $T_4$ on pituitary thyrotropes) and yet an additional *ultrashort* loop, more recently described, which also occurs at the pituitary gland through actions of locally secreted TSH (fig. 1a).

*Long Negative Feedback Loop*

The physiology of TRH is controlled by thyroid hormones. $T_4$ reaches the TRH-producing PVN neurons at the hypothalamus by two pathways: directly crossing the blood-brain barrier through known thyroid hormone transporters (organic anion-transporting polypeptide 1C1, OATP1C1), and crossing the cerebrospinal barrier to access the cerebrospinal fluid. From the cerebrospinal fluid, $T_4$ enters PVN neurons indirectly through tanycytes, glial cells located at the third ventricle wall expressing the monocarboxylate transporter 8 (MCT8) thyroid hormone transporter. Once within tanycytes, DIO2 activates $T_4$ to $T_3$, which finally enters PVN neurons and exerts negative transcriptional effects on the *TRH* promoter. Therefore, DIO2 regulates the intracellular availability of $T_3$ in PVN neurons, mediating the regulation of the thyroid axis. PVN neurons also express DIO3, which degrades $T_3$ to inactive products. Since DIO3 expression is stimulated by (excess) $T_3$, overall these data suggest the existence of a local control mechanism to compensate for possible variations in the $T_3$ intracellular availability [35–37].

Yet another regulatory step is the stimulation of pyroglutamyl peptidase II (PPII) by $T_3$ in tanycytes: these cells also interact with axon terminals of the PVN hypothalamic neurons and degrade released TRH excess at the median eminence. Therefore, the long feedback loop principally involves the transcriptional inhibitory effects of $T_3$ on the *TRH* under a substantial modulation by local thyroid hormone transporters, deiodinases and peptide-degrading enzymes [18, 22].

*Short Negative Feedback Loop*

Thyroid hormones potently downregulate TSH production by thyrotropic cells. $T_4$ (and to a lesser extent also $T_3$) reaches the thyrotropes through the systemic circulation, enters these cells by still poorly defined thyroid hormone transporters and must be deiodinated to $T_3$ by DIO2, the predominant deiodinase in the pituitary. In contrast to the hypothalamic PVN neurons, thyrotropes do not express DIO3. $T_3$ then reaches the nucleus and binds thyroid hormone receptor β (TRβ), being the predominant component of the negative transcriptional regulation of the *TSHB* promoter. The major isoform of TRβ in the pituitary is $TR\beta_2$, but the $TR\beta_1$ isoform is also residually present. The $T_3$-$TR\beta_2$ complex interacts with thyroid response elements located in the *TSHB* promoter, ultimately repressing gene expression (fig. 1c) [38].

*Ultrashort Negative Feedback Loop*

A local TSH secretion control system was also identified involving a molecular dialogue between the thyrotropes and the folliculo-stellate cells from the anterior pituitary which, intriguingly, express the TSH receptor *(TSHR)*. In this circuit, TSH would



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be secreted into the extracellular space of thyrotropes and bind TSHRs of folliculostellate cells which would activate the JAK/STAT5a signaling pathway inducing expression of transforming growth factor (TGF)-$\beta_2$ (or other secreted paracrine factors) which, in turn, would stimulate TGFB receptors in thyrotropes leading to downregulation of TSH secretion [39] (fig. 1b). This paracrine and local autoregulatory effect of TSH may not only add to the classical negative feedback modulation of TSH secretion at the pituitary, but also be involved in TSH pulsatility, counteracting increased TSH levels after pulsatile TRH peaks [40].

*Pituitary Cell Plasticity and Transdifferentiation between Pituitary Cell Types*

A novel effect of thyroid hormone during pituitary embryogenesis has been identified in an animal model [41]. Before the onset of negative feedback regulation of thyrotrope function, excess $T_4$ causes thyrotope cell death. Interestingly, a slow recovery of thyrotrope cell mass occurs following removal of excess $T_4$. This regulation of thyrotrope development by $T_4$ may have important implications for the functional reserve of TSH production and the TSH set point later in life. Actually transient CCH is described in children born from mothers with gestational hyperthyroidism. Transiency of this phenotype and the recovery of thyrotrope numbers after cessation of $T_4$ excess points towards a relevant plasticity of the pituitary tissue, which may involve transdifferentiation between different pituitary cell types when necessary, in this particular case from somatotropes towards thyrotropes [42, 43].

*Hypothalamic Dopamine and Somatostatin*

Apart from thyroid hormones themselves, *TSHB* expression is subject to negative regulation by hypothalamic peptides. Somatostatin and dopamine are neurotransmitters secreted by the hypothalamus acting on different pituitary cell types including thyrotropes, somatotropes and lactotropes. They bind to specific GPCRs and negatively regulate the expression of *TSHB.* Dopamine acts through the D2 receptor in the pituitary [44], whereas somatostatin uses somatostatin receptors (SSTR) 1, 2, 3 and 5. TSH secretion is inhibited through SSTR2 and -5 [45].

Stimulation of these receptors triggers inactivation of adenylate cyclase and phospholipase C. Adenylate cyclase inactivation negatively modulates the amount of cAMP and a decrease in *TSHB* expression [44, 45] counteracting the TRH-TRHR pathway.

The D2 receptor additionally inhibits the expression *POU1F1*, further downregulating the expression of *TSHB* and prolactin [46]. A divergent action is however mediated between dopamine receptors and somatostatin receptors on the phospholipase C signaling route. The dopamine D2 receptor inactivates phospholipase C, preventing calcium efflux to the cytosol and decreasing TSH release to the extracellular space [44]. However, somatostatin-somatostatin receptors produce the opposite effect [45]. Similar to the TRHR, both dopamine receptors and somatostatin receptors are also



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subject to its internalization and desensitization mechanisms [45]. Dopamine is widely used in pediatrics as intensive care support in cardiovascular dysfunction, and somatostatin analogs are indicated for pituitary growth hormone (GH)-secreting adenomas. Both can produce iatrogenic central hypothyroidism in the hospitalization/pharmacological setting.

*Other Signals Involved in Thyroid-Stimulating Hormone Action: Thyrostimulin*

Thyrostimulin is a novel glycoprotein hormone composed of 2 subunits, $\alpha_2$ (encoded by *GPA2*) and $\beta_5$ (encoded by *GPB5*) which strongly activates the TSHR [47]. It is allegedly undetectable in serum; therefore it is unlikely to form part of the classic endocrine negative-feedback regulation of the hypothalamic-pituitary-thyroid axis. However, GPA2 and GPB5 colocalize in the anterior pituitary, where TSHR is also present, and a paracrine regulatory role on the TSH secretion was suggested [39, 40]. However, the putative biological role of the heterodimer remains a matter of debate, including the possibility of independent actions for each monomer. Mice overexpressing *GPB5* show mild hyperthyroxinemia [48]. In contrast, knockout mice of *GPB5* show transient juvenile hypothyroxinemia of unclarified origin [49]. Recently, *GPB5* was shown to be positively regulated in vitro by inflammatory cytokines (tumor necrosis factors or interleukins) [50]. Furthermore, in a classical model for nonthyroidal illness, hypothalamic and pituitary GPB5 is dramatically increased in mice challenged with the administration of bacterial endotoxins inducing acute inflammation [51]. Animals presented typical profiles of nonthyroidal illness (low $T_4$ and $T_3$, low TSH) and a concomitant decrease in pituitary-hypothalamic TSHR mRNA, which was not present in *GPB5*$^{-/-}$ mice. These findings show differential in vivo upregulation of *GPB5* (and not *GPA2*) suggesting a distinct role of this component of thyrostimulin in central TSHR suppression during acute illness.

## *Molecular Actors Controlling Thyroid-Stimulating Hormone Action*

### *At the Thyrotropin-Releasing Hormone Gene Promoter*

Positive Transcriptional Control

TRH production is transcriptionally stimulated by the presence of regulatory elements in the *TRH* promoter, where factors such as cAMP response element binding protein (CREBP) and cAMP response element modulator (CREM) bind and transactivate *TRH* [52] (fig. 2a). These factors are the final mediators of a cAMP pathway derived from stimulation of a GPCR located at the cell membrane of PVN neurons: the melanocortin (MSHα) receptor 4. The latter is stimulated by MSHα, produced and secreted from the neighboring neurons of the arcuate nucleus from its natural precursor pro-opiomelanocortin. Yet another strong stimulus of *TRH* transcription is cold, whose molecular mediation is less well understood [53].



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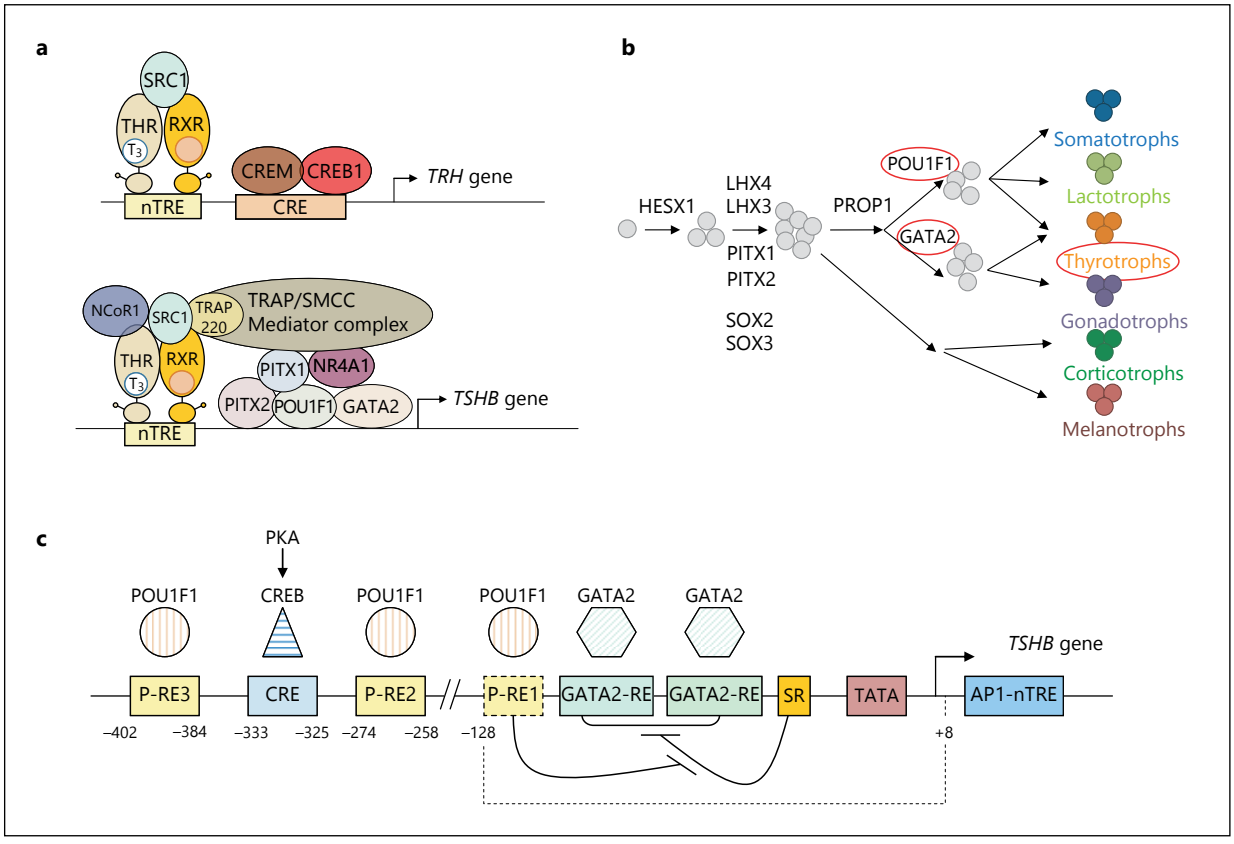


**Fig. 2.** Transcription factor involved in pituitary development and expression of *TRH* and *TSHB* genes. **a** *TRH* and *TSHB* transcriptional control. CREBP1 (cAMP response element binding protein 1) and CREM (cAMP response element modulator) bind promoter DNA and activate *TRH* transcription. The complex system of thyrotropin *(TSHB)* transcription factors includes POU1F1, GATA2, PITX1, PITX2, NR4A1, TRAP/SMCC mediator complex, steroid receptor coactivator 1 (SRC1) and nuclear receptor corepressor (NCoR1). Negative transcriptional control of *TRH* and *TSHB* is performed by heterodimers between retinoid X receptors (RXR) and thyroid hormone-T3 complexes (TR-T3) that interact with thyroid hormone response elements (TRE) of the promoters. **b** Transcription factors in development and function of different pituitary cell lines. These pituitary factors are expressed in a coordinated manner over time and space. The later they are expressed in pituitary development, the more specific to particular cell lines they generally are. POU1F1 and GATA2 are the two main essential factors for TSH synthesis in thyrotropes. **c** Detailed scheme on the binding of POU1F1 and GATA2 to the *TSHB* promoter. POU1F1 binds to P-RE2 (POU1F1 response element 2), P-RE3 and yet to other sites (termed here P-RE1) between –128 and +8 with different effects. P-RE2 and P-RE3 are located close to a CRE (cAMP response element) site, and P-RE1s are next to a GATA2-RE (GATA2 response element) partially avoiding SR (suppression region)-mediated inhibition of GATA2.



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Negative Transcriptional Control

*TRH* is one of the genes subject to negative modulation by $T_3$. In PVN neurons, $T_3$ is the most powerful inhibitory signal over the synthesis and secretion of TRH through binding to the $TR\beta_2$. Nuclear $TR\beta_2$-$T_3$ complexes interact with thyroid hormone response elements of the *TRH* promoter triggering the recruitment of corepressors or coactivators such as steroid receptor coactivator 1 (SRC1) necessary for repression of *TRH* [54] (fig. 2a). However, other powerful signals negatively control *TRH*. During fasting, arcuate nucleus neurons produce neuropeptide Y (NPY) that exerts a negative effect over TRH synthesis through its action on two different hypothalamic nuclei: arcuate nucleus neurons producing MSHα and PVN neurons producing TRH. NPY posttranscriptionally inhibits MSHα's precursor pro-opiomelanocortin by decreasing endopeptidase proconvertase 2, impairing proteolytic generation of pro-opiomelanocortin-derived active peptides. Furthermore, NPY stimulates its specific receptor in PVN neurons, which unfolds an inhibitory cascade on *TRH* transcription through activation of inhibitory G proteins leading to a decrease in intracellular cAMP antagonizing the central effects of MSHα in arcuate nucleus and PVN. NPY also downregulates endopeptidase proconvertase 2 in the TRH neurons, reducing pro-TRH processing [55]. Therefore, NPY (and food intake) appears to be a master regulator of TRH secretion via 3 distinct mechanisms: (1) reducing *MSHα* expression, the main external stimulus for TRH synthesis, (2) inhibiting *TRH* transcription in the PVN through the NPY receptor, and (3) reducing the amount of mature TRH by decreasing endopeptidase proconvertase 2.

*At the TSHB Gene Promoter*

Positive Transcriptional Control

TRH released from the hypothalamus stimulates TRHR located at the cell membrane of thyrotropes, signaling through a GPCR pathway that activates kinases which phosphorylate factors whose activation ultimately favors transcription of the *TSHB* gene (fig. 1c). A complex system of transcription factors stimulating *TSHB* expression includes GATA2, POU1F1, PITX1, PITX2, NR4A1, TRAP220 and nuclear receptor corepressor 1 (NCOR1) and other nuclear proteins (fig. 2a). GATA2 has emerged as the main mediator in transcriptional activation of *TSHB*. This factor is essential not only for *TSHB* transcription, but also for the synthesis of gonadotropic hormones (follicle-stimulating hormone, luteinizing hormone). However, *TSHB* necessarily requires functional cooperation between GATA2 and POU1F1. POU1F1 (also known as PIT1) is a homeodomain transcription factor with a specific POU domain and a DNA binding domain. Both domains are important for activation of *TSHB*, *GH* and prolactin promoters at thyrotropes, lactotropes and somatotropes, respectively. As for the *TSHB* promoter, POU1F1 binds at 3 different sites P-RE2 and P-RE3 (located at –274/–278 and –402/–384, respectively), near a cAMP response element that contributes to *TSHB* expression mediated by the TRH-TRHR pathway. P-RE1 is a poorly defined POU1F1-responsive element (between nucleotides –128 and +8) in the vicin-



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ity of two GATA2 response elements that help protecting GATA2 from inhibition by a suppressor region encompassing the downstream region of GATA response elements (fig. 2c) [56, 57]. The presence of different P-REs and their functional interaction with GATA2 response elements reflects the critical importance of POU1F1 in the transcriptional modulation of *TSHB*.

PITX2 and PITX1 are two homologous transcription factors expressed in the pituitary with similar and complementary functions (but differentially expressed during development) contributing to the formation of Rathke's pouch (future pituitary gland) and other organs like the heart, eye and oronasal cavities. In adults, PITX2 is relevant for thyroid function since it regulates the expression of *TSHB* and other transcription factors (POU1F1, PROP1, LHX3) necessary for *TSHB* transcription [58]. PITX1 is involved in the response of the pituitary against hypothyroidism, inducing an increase in the biosynthesis and secretion of TSH when thyroid hormone levels are low. Although for optimal response PITX2 is necessary, PITX1 can supply the minimal necessary stimulus for *TSHB* transcription in mouse models [59].

Other factors involved in the thyrotropin synthesis are NR4A1 (Nur77) and TRAP/SMCC complexes (thyroid hormone receptor-associated protein complex, alternatively named mediator complex). The NR4A1 orphan nuclear receptor is expressed in thyrotropes but also in corticotropes and gonadotropes, and its expression is positively regulated by the signaling cascade triggered by TRH-TRHR. In vivo studies show that NR4A1 activates transcription of *TSHB*, constituting a regulating member of the hypothalamus-pituitary-thyroid axis. It is believed that this factor acts cooperatively with POU1F1 and GATA2 on *TSHB* transcription [60]. The TRAP/SMCC complexes are composed of 30 common subunits highly conserved in the animal kingdom, being part of the transcriptional regulatory machinery in many cell types, upregulating the expression of genes through interaction with a specific nuclear receptor. TRAP220 (also known as MED1) favors pituitary TSHB synthesis, but its precise mechanism of action is obscure. It may interact with the TR in a ligand-independent manner or, alternatively, act as a coactivator of factors positively involved in transcription, such as GATA2 and POU1F1. Regardless of the action mechanism, the TRAP complex plays an important role in maintaining homeostasis of thyroid hormones [61].

### Negative Transcriptional Control

$T_3$ also exerts the major negative control of the synthesis and release of TSH by binding to the TR and subsequent recruitment of nuclear coactivators or corepressors such as SRC1. In vivo studies have shown that NCOR1 by interaction with TR may play a regulatory 'bidirectional' (positive and negative) role over the expression of *TSHB* [62] (fig. 2a). Yet another mechanism of *TSHB* negative transcriptional regulation is suggested to exist from TGFB receptor signaling in thyrotropes. However to date, this mechanism remains poorly investigated [39, 40].



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## Animal and Cellular Models of Central Hypothyroidism

The genetic basis of human CCH is largely unknown, especially in case of hypothalamic CH. However, various cellular or animal models have been generated to better understand hormonal routes and molecular signaling altered in CCH and identify patients with clinical symptoms and phenotypic traits present in such models, helping to define phenotype-genotype correlations in this group of disorders. Table 1 provides currently available preclinical in vivo models that implicate genes in abnormalities of the central control of the thyroid hormone axis, including the respective protein functions and details of the various hypothyroid (sometimes compensatorily euthyroid) phenotypes. Genetic models for combined pituitary hormone deficiency are also included when the thyroid axis is affected.

## Central Hypothyroidism in Pediatrics

### *Identification, Etiological Investigations and Clinical Management*

Diagnosis and therapeutic management of central hypothyroidism pose clinical challenges in pediatric and adult endocrinology [80, 81]. In countries using sophisticated $T_4$-based CH screening strategies ($T_4$/TSH/TBG or $FT_4$/TSHs), neonates with CCH may be reliably detected, etiologically investigated and treated early. These strategies prevent any mental retardation derived from neonatal-infantile hypothyroidism. Life-threatening hypoglycemias will also be prevented in case CCH is part of combined pituitary hormone deficiencies with failing adrenocorticotropic hormone or GH, which would also be investigated, diagnosed and treated promptly as part of the study of a baby with central hormonal failure; diagnosis of severe CCH usually occurs at 2–8 months of age, associating mental deficits [7–9]. Milder CCH forms are typically diagnosed by chance in children who may present with short stature [82] and unspecific symptoms compatible with hypothyroidism.

Diagnosis of CCH requires a high degree of clinical suspicion. Children may present either with mildly decreased $T_4$ and low-normal or sometimes mildly elevated TSH when the predominant origin of CCH is pituitary or hypothalamic, respectively. In this setting, interpretation of hormonal results is difficult, and calculation of the TSH index may be of value [83]. This index is based on the logarithmic standard model of thyroid homeostasis and may help predicting the risk of failure of thyrotropic function. An alternative approach based on the linear comparison of TSH and $FT_4$ consists in plotting both values on a chart containing percentiles of pituitary 'response' [19]. The latter considerably overlaps with the interpretation of hormone levels by conventional reference values; however, it may more easily detect significant deviations compatible with central hypothyroidism. Given the difficulty of diagnosis, determination of indexes of peripheral thyroid hormone action like sex hormone-binding globulin (SHBG), serum lipids or others, may



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**Table 1.** Animal and cellular models of central hypothyroidism and central dysregulation of the thyroid axis: manipulated genes, protein function and associated hormonal phenotypes

| Model | Gene | Function | Hormonal profile and associated features | Ref. No. |
|---|---|---|---|---|
| *Hypothalamic central hypothyroidism* | | | | |
| –/– KO mouse | *Trh* | Thyrotropin synthesis, bioactivity and release from pituitary | ↑ sTSH with ↓ bioactivity, ↓ $sT_4$<br>Hyperglycemia | 63 |
| –/– KO mouse | *Pcsk1* and *Pcsk2* | Endopeptidase that generates mature TRH peptides from pro-TRH | ↓ mature TRH peptides,<br>↓ $sT_3$ (*Pcsk1* KO), N $sT_3$ (*Pcsk2* KO) | 64 |
| Rat under PPII inhibitor | *PPII* | Peptidase that degrades and deactivates TRH | ↑ sTSH (under TRH and cold stimulation) | 22 |
| –/– KO mouse in PVN* | *Creb1* | Transcription factor: positively regulates TRH synthesis | N sTSH, N $sT_4$, N $sT_3$, compensatory ↑ mRNA *Trh* (by ↑ mRNA *Crem*) | 52 |
| Deficient Ob/Ob mouse | *Lep* | Regulating expression of pro-TRH and proconvertases 1 and 2 | Hypothalamic hypothyroidism | 65 |
| *Pituitary central hypothyroidism* | | | | |
| –/– KO mouse | *Trhr1* | Mediates TRH effects in thyrotropes | N sTSH, ↓ $sT_4$, ↓ $sT_3$, N sPRL | 66 |
| –/– KO mouse | *Cga/Gsu* | α-Subunit component of TSH, LH and FSH | Absence of sTSH, $sT_4$, prot. FSH and prot. LH<br>Hypogonadism, infertility, dwarfism | 67 |
| IS mouse<br>Snell dwarf | *Pou1f1/ Pit1* | Transcription factor: positively regulates TSH, PRL and GH synthesis | ↓ prot. TSH, ↓ prot. GH, ↓ prot. PRL<br>Dwarfism | 68 |
| Pituitary –/– KO mouse* | *Gata2* | Transcription factor: positively regulates TSH, FSH and LH synthesis | ↓ sTSH, ↓ sFSH<br>Maintain fertility | 69 |
| +/– KO mouse | *Trap220/ Med1* | Transcriptional coactivator | ↓ mRNA *Tshβ*<br>Growth retardation | 70 |
| –/– KO mouse | *Gphb5* | Glycoprotein hormone subunit of thyrostimulin activates the TSH receptor | N sTSH, ↓ $sT_4$ (transiently in juvenile mice)<br>Euthyroidism in adult mice | 49 |
| –/– KO mouse# | *Igsf1* | Possible membrane receptor involved in pituitary regulation | ↓ sTSH, ↓ $sT_3$, ↓ mRNA *Trhr*<br>Overweight, fertile (X-linked phenotype) | 12<br>71 |
| –/– KO mouse | *Prop1* | Transcription factor involved in pituitary cell differentiation and organogenesis | ↓ prot. TSH, ↓ prot. GH, ↓ prot. PRL, ↓ prot. FSH, ↓ prot. LH<br>Pituitary hypoplasia, dwarfism, hypogonadism | 72 |
| –/– KO mouse | *Lhx3* | Transcription factor involved in pituitary organogenesis | Absence of prot. TSH, prot. GH, prot. LH and prot. PRL<br>Absence of mRNA *Pou1f1*, ↓ mRNA *TPit*<br>Lethal, pituitary hypoplasia | 73 |
| –/– KO mouse | *Lhx4* | Transcription factor involved in pituitary organogenesis | ↓ prot. TSH, ↓ prot. GH, ↓ prot. LH, ↓ prot. PRL,<br>↓ prot. POMC Lethal, pituitary hypoplasia | 74 |
| –/– KO mouse | *Pitx1* | Transcription factor involved in pituitary organogenesis | ↓ prot. TSH, ↓ prot. LH, ↓ prot. FSH<br>Lethal, hind limb abnormalities | 75 |
| Hypomorphic PITX2 mouse | *Pitx2* | Transcription factor involved in pituitary organogenesis | ↓ prot. TSH, ↓ prot. GH, absence of prot. FSH and prot. LH, ↓ mRNA *Gata2*, ↓ mRNA *Pou1f1*; lethal at birth | 76 |
| +/– KO mouse | *Sox2* | Transcription factor involved in pituitary, CNS and placode development | ↓ prot. TSH, ↓ prot. GH, ↓ prot. LH, ↓ prot. PRL<br>Rathke's cleft, impaired male fertility, hypogonadism, dwarfism | 77 |



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**Table 1.** Continued

| Model | Gene | Function | Hormonal profile and associated features | Ref. No. |
|---|---|---|---|---|
| –/– KO mouse | *Sox3* | Transcription factor involved in pituitary and CNS midline development | ↓ prot. TSH, ↓ prot. GH, ↓ prot. LH, ↓ prot. FSH Rathke's cleft, hypogonadism, dwarfism, hypothalamic defects (X-linked phenotype) | 78 |
| KO mouse | *Hesx1* | Transcription factor involved in pituitary, optical nerve and brain development | Rathke's cleft, brain and eye dysplasia, agenesis of corpus callosum and septum pellucidum | 79 |

KO = Knockout; IS = inbred strain; –/– = homozygous inactivation; +/– = heterozygous inactivation; * = tissue-specific; # = incomplete inactivation: IGSF1 variant 4 mRNA isoform remains expressed; N = normal; ↑ = elevated; ↓ = diminished; s = serum levels; prot. = tissue protein levels; CNS = central nervous system; PRL = prolactin; FSH = follicle-stimulating hormone; LH = luteinizing hormone; POMC = pro-opiomelanocortin; *Trh* = thyrotropin-releasing hormone; *Pcsk* = prohormone convertase; *PPII* = pyroglutamyl peptidase II; *Creb1* = cAMP response element binding protein 1; *Crem* = cAMP response element modulator; *Trhr1* = thyrotropin-releasing hormone receptor 1; *Cga/Gsu* = glycoprotein hormone α-subunit; *Pou1f1/Pit1* = pituitary-specific positive transcription factor 1; *Gata2* = Gata binding protein 2; *Trap220/Med1* = mediator complex subunit 1; *Gphb5* = glycoprotein hormone β-subunit 5; *Igsf1* = immunoglobulin superfamily factor 1; *Prop1* = homeobox protein prophet of *Pit1; Lhx3* and *Lhx4* = LIM/homeobox proteins; *Pitx1* and *Pitx2* = pituitary homeobox 1 and 2; *Sox2* and *Sox3* = SRY (sex-determining region Y) box 2 and 3; *Hesx1* = HESX homeobox 1. Rat under PPII inhibitor *HcPI* (*Hermodice carunculata* protease) and injected with TRH or exposed to a cold environment. X-linked = Gene in X chromosome, phenotype X linkage.

support the existence of CCH [80]. In this line, recent reports suggest that $T_3$-dependent parameters of Doppler echocardiography such as the isovolumic contraction time, isovolumic contraction time/ejection time and myocardial performance index correlate well with the presence of CCH in adult patients with hypothalamic-pituitary diseases, thus suggesting a diagnostic potential for 'hidden' CCH [84], yet unexplored in children. Although not frequently performed since it requires hospitalization, evaluation of the nocturnal TSH surge may be useful in the diagnosis of milder forms of CCH [85, 86]. The TRH test may also confirm the suspicion of mild CCH and be informative in the differential diagnosis of secondary (pituitary) versus tertiary (hypothalamic) CCH [80]. Some authors questioned the utility of this test arguing the existence of overlapping TSH responses between hypothalamic and pituitary defects [87, 88]. However, others claim it is useful to estimate the biological activity of circulating TSH through the increment of $T_3$ in response to increased TSH [81]. To maximize the diagnostic efficiency of the test on discrimination between hypothalamic and pituitary disease, it is suggested to carry out a test lasting 180 min [89]. The TRH test is based on the determination of serum TSH at different times after intravenous administration of 7 μg/kg TRH (10 μg/kg in babies, with higher distribution volume). Evaluation of the test focuses on magnitude and timing of TSH responses (TSH peaks occur normally at 15–30 min and should exceed increments of 4–5 mU/l (>15 mU/l in neonates [89]); lower increments suggest pituitary hypothyroidism). Delayed and excessive TSH peaks together with lack of complete recovery to basal TSH levels at 180 min suggest a hypothalamic defect. MRI of the hypothalamic-pituitary area is required in most suspected cases of CCH to detect the origin of the disorder. To complete the battery of tools available to study patients with suspected CCH, serum of these patients can be investigated in vitro by cell-based assays to determine the



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bioactivity of the TSH against the TSHR, known to be mediated by the glycosylation pattern of the TSH dimer which, in turn, is under tight control of TRH signaling.

Proper identification of children and adults with CCH is important, but pregnant women with CCH require special attention. They may develop gestational hypothyroxinemia, which can be missed by routine TSH evaluation of the thyroid axis during pregnancy. This 'unnoticed' low $T_4$ in the mother can aggravate the hypothyroid state of the fetus who may well have inherited the genetic defect from the mother, overall resulting in short supplies of thyroid hormone to support normal fetal brain development, leading to severe CH with the consequences of gross motor and speech delay [90].

As for the treatment, neonates with CCH require full replacement doses at 10–15 μg/kg daily of levothyroxine, with progressive reduction of relative doses required. Circulating $FT_4$ level is the parameter to guide and monitor doses of levothyroxine, since TSH is normally completely suppressed after initiation of therapy. Although TRH and TSH would be theoretically ideal for the treatment of CCH, they were abandoned in favour of L-T4 because of their low stability at oral administration, limited applicability and high costs, but in the past TRH was successfully used in hypothalamic CCH patients.

## Clinical Forms of Human Central Hypothyroidism

A classification of clinical forms of central hypothyroidism in humans can be based on its *genetic* (permanent) or *nongenetic* etiology (transient/reversible).

### *Genetic Central Hypothyroidism*

#### *Isolated Thyroid-Stimulating Hormone Deficiency*

##### TRHR Gene Defects

The TRHR mediates TRH signaling in thyrotropes towards the synthesis, secretion and correct glycosylation of TSH. Only 3 human cases from 2 pedigrees have been described with mutations in the *TRHR* gene, exhibiting autosomal recessive inheritance [91, 92]. Index patients were children of 9 and 11 years from unrelated families with very similar clinical presentation consisting of short stature and variable symptoms suggestive of hypothyroidism like fatigue, lethargy or poor school performance. They were born in Canada and Italy, respectively, both countries using TSH-based screens for CH, therefore the existence of neonatal hypothyroidism could not be confirmed. Both patients were fortuitously diagnosed with hypothyroidism in the course of clinical investigations for short stature. They both presented markedly delayed bone ages (2.5 and 4.5 years with respect to chronological age, respectively). Hormonal profiles revealed thyrotropin levels within the normal range (0.6 and 1.3 mU/l, respectively) with suspected low bioactivity, as indicated by the presence of mild to frank hypothyroidism ($T_4$: 4.0 μg/dl,



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normal: 4.5–11.5; $FT_4$: 0.36 ng/dl, normal 0.9–1.7, respectively). In both cases, TRH tests showed no response of TSH and prolactin. Short stature may be attributable to hypothyroidism during infancy since height velocity was reported to increase after levothyroxine replacement in 1 case [91] or allow reaching parental target height in the other [92]. No obvious cognitive or psychomotor deficits were reported for these patients, in contrast to the severe cretinism affecting children with *TSHB* gene defects [93] suggesting that, if present, hypothyroidism had to be mild during the first few years of life.

As for the genetic defects identified, the Canadian patient was compound heterozygote for 2 different mutations in *TRHR*, consistent with recessive heredity of the disease. In his paternal allele, the patient harbored a premature stop codon R17X, which fully inactivates protein function. On the maternal allele, he had a complex combination of mutations: 9-nucleotide deletion followed by a point mutation, resulting in an in-frame deletion of 3 amino acids (Ser115-Thr117) plus a missense change (Ala118 for Thr118; p.S115-T117del+T118) located at the cytoplasmic end of the third transmembrane domain of the receptor (fig. 3a) [91].

The Italian patient was homozygote for the mentioned early nonsense mutation in *TRHR* (p.R17X; fig. 3a) [92]. Interestingly, a female patient from the Italian pedigree with biallelic mutation was diagnosed with hypothyroidism in adulthood. She also had hypothyroxinemia but had become pregnant twice and delivered healthy term babies who were breast-fed, suggesting TRH is not essential for human lactation. All in all, the mildness of this phenotype strongly suggests a clinical underdetection of human *TRHR* defects and anticipates that non-TRH signals may contribute to the TRH-TRHR signaling in thyrotropes, especially since circadian biorhythms of TSH, prolactin and cortisol secretion were shown to be conserved in this condition.

As for possible other phenotypes derived from impairment of TRH-TRHR signaling, mutations in the *TRH* gene have not been identified in humans. However, TRH-deficient mice show hypothalamic hypothyroidism characterized by mild elevation of TSH levels with reduced TSH bioactivity [63]. In this model, the lack of TRH stimuli at the pituitary causes a decrease in TSH-producing cell mass. Such mice also showed extrathyroidal features like hyperglycemia associated with impaired insulin secretion in response to glucose. As for other examples, the murine *TRH* knockout mouse model may guide the identification of still unedited human phenotypes linked to *TRH* gene defects. Similarly, the leptin knockout model mice (*ob/ob*) present central hypothyroidism of hypothalamic origin [65]. In humans, such hypothyroidism is not well characterized but it is reported to be also hypothalamic, not being present in children but in adulthood and be presumably reversible by leptin substitution treatment [31].

### TSHB Gene Defects

Most reported cases of isolated CCH correspond to mutations in the β-subunit of *TSH*, causing the most severe phenotype of central hypothyroidism known to date.

Like other family members of the glycoprotein hormones, TSH is a dimeric cystine knot protein composed by α- and β-chains. In the *TSHB* gene, 9 different mutations



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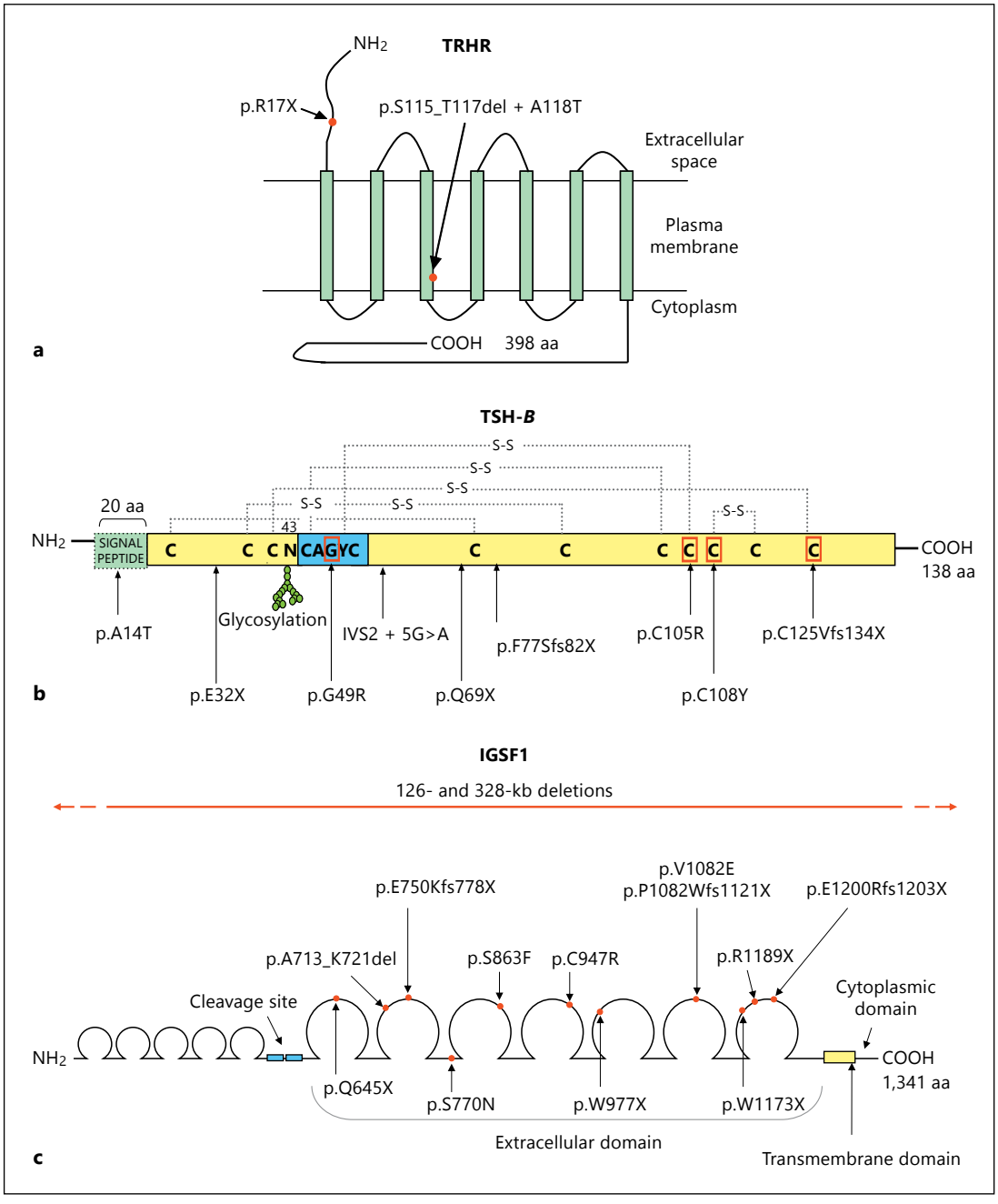


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have been described leading to an isolated deficiency of TSH of autosomal recessive inheritance [7–9, 93–99].

Since neonatal CH screening programs in most countries rely on elevations of TSH, patients with defects in *TSHB* frequently have a delayed diagnosis. Most are clinically detected later than 3 months from birth (3–8 months), revealing a neuromo-



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tor and mental retardation in psychometric evaluations at 2–8 years of age. In contrast, patients exceptionally diagnosed and treated within the first weeks of life by symptoms of hypothyroidism avoid mental retardation [99]. All cases present blunted responses of TSH after TRH administration but the response to prolactin is completely normal.

The most frequent mutation in *TSHB* is a deletion of 1 nucleotide at codon 125 of exon 3 (c.T373del; p.C125Vfs134X; former nomenclature: c.T313del; p.C105Vfs114X; explanation in footnote for fig. 3b) that leads to a frameshift and a prematurely truncated protein (fig. 3b). Such an aberrant protein may not be detectable in routine TSH immunoassays. However, it is detectable by some TSH assays in patients showing insufficient (but present) TSH response at the TRH test. Cell-based in vitro studies have shown that this mutation leads to a lower bioactivity of the TSH dimer [94]. Based on crystallography of chorionic gonadotropin, this mutation may disturb a disulfide bond formed between cysteines 39 and 125, which conforms a 'buckle' or a 'seat belt' surrounding the α-subunit monomer common to glycoprotein hormones.

Another frequent and deleterious mutation in *TSHB* found in patients with isolated TSH deficiency and cretinism is the amino acid substitution G49R (initially described as G29R), located in the CAGYC region of the protein (fig. 3b) [93]. A 3-dimensional imaging analysis of the TSH dimer revealed that the CAGYC region is important for heterodimerization of β- and α-chains of TSH, which is only active as a dimeric molecule. Patients with this mutation not only have undetectable TSH in serum, but also strongly decreased or undetectable $T_4$ and $T_3$ concentrations consistent with the severe biochemical and clinical hypothyroidism linked to this genotype.

A detailed clinical description of typical *TSHB* defects was reported from a baby girl of 75 days of age referred for severe symptoms of hypothyroidism [8]. Characteristic features of the condition were completed with the existence of a hyperplastic pituitary gland (reversible after some years of $T_4$ treatment), extremely high levels of circulating serum α-subunit and a hypoplastic thyroid gland incapable of any $^{99}Tc$ uptake. The patient harbored the nonsense mutation Q69X (originally annotated as

**Fig. 3.** Mutations identified in proteins causing isolated CCH. **a** TRHR or thyrotropin-releasing hormone receptor is a transmembrane protein in which only 2 mutations have been described: one in the extracellular domain and another in the third transmembrane domain. **b** TSH-β or thyrotropin β-subunit is a cystine knot protein containing a signal peptide of 20 amino acids, the CAGYC conserve heterodimerization domain between β- and α-chains and arginine 43 that is glycosylated and involved in modulation of TSH biopotency. Nine different mutations have been identified in this protein, p.C125Vfs134X being the most frequent. The nomenclature of these mutations follows the modern 'guidelines' (den Dunnen et al. [121]), including the N-terminal 20 amino acids of the signal peptide (this may cause differences in description of mutants in the past between old articles published and the current database HMC). **c** IGSF1 is a plasma membrane immunoglobulin superfamily glycoprotein containing 12 C2-type immunoglobulin loops, a transmembrane domain and an intracellular tail. This protein is cleaved at the hydrophobic linker between the 5th and 6th immunoglobulin loops. All mutations so far described are located in the extracellular domain.



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Q49X) in TSHB (fig. 3b) deleting 60% of the C-terminal amino acid sequence, in spite of which the mutant was able to form heterodimers with the α-subunit as reflected by its preserved immunoreactivity (TSH was documented normal in serum). The mutant dimer was however completely devoid of bioactivity.

Six additional mutations have been described to date in *TSHB* corresponding to nonsense (p.E32X), frameshift (p.F77Sfs82X), splice site (IVS2 + 5G>A) and missense mutations (p.A14T, p.Q69X; p.C105R, p.C108Y) [7, 9, 95–98]. As for the pathogenic mechanisms for these mutations, lack of TSH secretion may be involved for p.A14T, a mutation located at the signal peptide of the protein and, interestingly, the rest of missense mutants cause changes of cysteine residues (C) involved in the formation of disulfide bonds critical for the formation of 'loops' characteristic of the tertiary structure of cystine knot proteins (fig. 3b).

*IGSF1* Gene Defects

In 2012, a novel gene named *IGSF1* (formerly known as InhBP/p120) involved in human central hypothyroidism was identified [12]. The gene is located in chromosome X, and *IGSF1* defects affect mainly males, but also female cases were recently described with central CH [15]. Strikingly, male patients also show macroorchidism of unclear pathogenesis and undetermined time of onset. The association of hypothyroidism and macroorchidism was classically described in the pre-screening CH era in boys with long-term undiagnosed hypothyroidism [100]. The increased size of the testicles in such cases was due to cross-stimulation of the testicular follicle-stimulating-hormone receptor by chronically and intensely elevated TSH in these boys [101]. However, *IGSF1* defects show TSH deficiency, and such pathogenic mechanism cannot be claimed for this frequently (but not invariably) associated feature of the disorder [13]. It was not until recently when the association of human central hypothyroidism and macroorchidism was clinically described in 2009 in 2 patients with isolated TSH deficiency and prepubertal testicular enlargement [102].

Hypothyroidism in this X-linked disease is more severe in males, while in females a milder hypothyroidism with normal growth and pubertal development was described [15]. Patients can variably show additional pituitary defects in prolactin and GH, which can be partial [12–15]. Delayed puberty and overweight have also been described in patients.

So far, 18 cases from 16 pedigrees have been detected with *IGSF1* defects. Most patients were diagnosed in the Netherlands and Japan, both countries using $T_4$-based screening CH programs ($T_4$/TSH/TBG strategy or $FT_4$/TSH schemes, respectively) showing low (or borderline low) $T_4$ and normal TSH. Most Dutch cases (13 patients of 11 families) were detected through neonatal screening. In serum, thyroid function tests showed TSH concentrations between 1.4 and 6.0 mU/l and decreased $T_4$ of 60–86% of the lower limit of reference. Three Japanese neonates were detected with a TSH between 1.2 and 2.7 mU/l and $FT_4$ between 0.68 and 0.79 ng/dl, while 2 patients (later identified) escaped neonatal detection with this method [13, 14].



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The disease is characterized by CCH of pituitary origin, showing diminished (but present) TSH responses in the TRH stimulation test.

In the Netherlands, macroorchidism was observed in all male patients from 12 years of age, together with delayed testosterone production. Serum follicle-stimulating hormone levels were higher than those of luteinizing hormone, and only in some cases did levels exceed the reference range. Inhibin-B and antimüllerian hormone were different among patients showing a tendency to be increased and decreased, respectively [12, 15].

No controlled psychometric evaluation of IGSF1-deficient patients detected at screening versus those diagnosed later has been performed. However, the described degree of neonatal hypothyroidism, when sustained through time, would usually leave the latter group of patients at risk of psychomotor retardation or intellectual deficits.

IGSF1 is a membrane domain protein with 12 typical immunoglobulin loops and a short cytoplasmic tail. When localized in the membrane, 7 immunoglobulin loops face the extracellular milieu and 5 of them are cleaved off (fig. 3c). The remaining protein is similar to a kinase membrane receptor but allegedly without kinase activity [103].

Mutations in 16 families have been identified disrupting the extracellular domain of the protein [12–15]. Two complete deletions and 12 mutations have been reported in *IGSF1*. Of these mutations, 5 were amino acid substitutions (p.A713_K721del, p.770N, p.S863F, p.C947R, p.V1082E), 4 nonsense mutations (p.Q645X, p.W977X, p.W1173X, p.R1189X) and 3 frameshift mutations producing a premature stop (p.E750Kfs778X, p.P1082Wfs1121X, p.E1200Rfs1203X; fig. 3c). So far, no clear genotype-phenotype correlations have been established between the type/localization of mutations and the clinical severity or time of onset of CCH.

*TSH Deficiency in the Context of Combined Pituitary Hormone Deficiency*

Embryological development of the pituitary gland is a complex process, resulting in the generation of 6 different cell lines (fig. 2b) through the interplay of timely and spatially coordinated action of diverse signaling pathways like Wnt/B-catenin, Sonic hedgehog, Fibroblast growth factors or Bone morphogenetic proteins [104, 105]. To this end, an expression cascade of early and late (terminal) differentiation transcription factors is necessary (fig. 2b), including factors specific to each pituitary cell type. There are transcription factors involved in the initial formation of the pituitary whose defects in humans result in complex phenotypes including central hypothyroidism such as HESX1, LHX3, LHX4, PITX2 and SOX3 [11] (fig. 2b). However, the generation of various lineages of the anterior pituitary requires the activity of several factors expressed later, responsible for the final differentiation of somatotropes, lactotropes, thyrotropes, gonadotropes, melanotropes and corticotropes. As for the particular case of thyrotropic differentiation, 3 transcription factors PROP1, POU1F1 and GATA2 are required [106]. Despite their proven involvement in pituitary organogenesis and thyrotrope differentiation in rodent models, human phenotypes for PITX1 and GATA2 defects are to date elusive (table 1). Recent reviews excellently summarize such pituitary phenotypes which are not the focus of the present update [11, 106].



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*Secondary to Gestational Hyperthyroidism*

Central hypothyroidism is highly prevalent in neonates born to women with uncontrolled hyperthyroidism during pregnancy. CH occurs in 1.5% of the offspring of women with Graves' disease when they are insufficiently treated during pregnancy, diagnosed after pregnancy or diagnosed after delivery [107].

Gestational hyperthyroidism induces a hyperthyroid fetal environment and exposure to high levels of thyroid hormone which affects the developing hypothalamus-pituitary-thyroid axis of the fetus. The onset of CCH in most such babies (62%) occurs a few days after birth. However, other neonates (33%) become hypothyroid after a euthyroid phase and, in rare cases (5%), after a short phase of hyperthyroidism caused by transplacental passage of stimulatory antibodies against the TSHR (thyroid hormone receptor antibodies, TR-Abs) to the neonate. CH children with CCH secondary to gestational hyperthyroidism present moderately to severely decreased $T_4$ combined with normal or suppressed TSH. The blunted TSH response to TRH administration confirms their impairment in their hypothalamus-pituitary-thyroid axis regulation [107] which could be explained by the stimulation of TR-Abs to the TSHR of pituitary folliculostellate cells involved in the negative ultrashort loop of TSH secretion [108].

Neonates who transiently need antithyroid drugs usually become euthyroid within 1–2 months, but their TSH levels remain suppressed for many months more yet under normal $T_4$ and $T_3$. Such TSH suppression could be eventually present until maternal TR-Abs are completely cleared by the baby [108]. This pathogenic mechanism was experimentally demonstrated in rats injected with TR-Ab-containing human IgG obtained from patients with Graves' disease, showing that TR-Abs are able to suppress TSH through pituitary pathways [109]. Moreover, it is known that Graves' disease patients with thyroid hormone inhibitory immunoglobulins have lower TSH values than patients without them. Although this is a convincing experimental mechanism to explain CCH in babies from mothers with Graves' disease, yet additional mechanisms may be active, including direct effects of $T_4$ excess over embryonic pituitary development. Recently, using zebrafish embryos Tonyushkina et al. [41] showed that early exposure to elevated $T_4$ leads to thyrotrope cell death at the beginning of embryonic development. Interestingly, such reduced thyrotropic cell mass tends to recover when the negative feedback regulation of the thyroid axis is established in the embryo, coinciding with expression of DIO3 in thyrotropes enabling conversion of (excess) $T_3$ to inactive hormones (reverse $T_3$ or diiodothyronine) [41]. The transient nature of central hypothyroidism in this animal model recalls the transiency of the described human disorder.

In the neonatal period the TSH response to TRH stimulation is abnormal; however, the axis is 'restored' within the first year of life and TH concentration remains within the normal range after withdrawal of $T_4$ treatment in most children [107]. Notwithstanding the above, some children may develop primary hypothyroidism as consequence of subtle thyroid abnormalities which are detectable by ultrasound.



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The outcome of CCH babies of mothers with gestational hyperthyroidism recalls the hyporesponsiveness of the pituitary TSH in adult patients with hyperthyroidism: after the start of antithyroid medication $T_4$ normalizes quickly, but the recovery of TSH secretion (both basal and in response to TRH) is rather slow, taking weeks or months [110, 111].

The transience of this particular CCH also supports the potential cellular plasticity of the pituitary. In this context, the multifunctional role of hormone-producing cells in the mouse pituitary has been described. Pituitary cells have a high plasticity, most cell types can transdifferentiate into any other pituitary cell, producing different hormones [42]. A specific cell type may well increase its mass by transdifferentiation, according to hormonal requirements. Indeed, a transdifferentiation of somatotropes into thyrotropes has been observed in patients with prolonged primary hypothyroidism [43].

*Iatrogenic (Pharmacological)*

Some medications may alter the thyroidal axis producing iatrogenic central hypothyroidism including frequently used antiepileptic drugs, dopamine or bexarotene. Antiepileptic medications such as valproic acid, carbamazepine or oxcarbamazepine increase the hepatic metabolism of thyroid hormones and their feedback effect at the pituitary, leading to central hypothyroidism [112]. Intravenous infusions of dopamine reduce TSH synthesis and secretion through inhibitory dopamine 2 receptor signaling in thyrotropes, especially in critically ill neonates and adults with nonthyroidal illness. Administration of the specific retinoid X receptor agonist bexarotene (used in patients with advanced cutaneous T cell lymphoma) decreases serum TSH, $FT_4$ and $T_3$, indicating inhibition of the *TSHB* promoter as plausible mechanism of action through activation of retinoid X receptor-TR heterodimers [113].

Other drugs like glucocorticoids and somatostatin reportedly suppress TSH synthesis and release but do not cause clinically significant central hypothyroidism. Chronic glucocorticoid administration inhibits TRH synthesis at the PVN through glucocorticoid response elements present at the TRH gene promoter, having a repressive effect when activated by the liganded glucocorticoid receptor. In contrast, acute and high doses of glucocorticoids are capable of significantly suppress serum TSH. Somatostatin analogs like octreotide, chronically administered in patients with acromegaly, induce a blunted TSH response to TRH and low $T_3$ despite normal basal TSH and $T_4$ levels, suggesting the drug alters thyroid hormone metabolism, which is consistent with the high levels of reverse $T_3$ identified in patients under such medication [114]. Of particular interest to pediatric endocrinology, GH replacement therapy was reported to induce reduction of TSH secretion in some patients [115] although it is controversial whether such central hypothyroidism could have yet been present (but not identified) before the initiation of GH treatment [116]. In any case, GH is capable of increasing peripheral deiodination of $T_4$ to $T_3$ (and thereby its biological activity) as well as induce somatostatin secretion, both mechanisms leading to reduction of TSH output from the pituitary [115]. Whether this effect is mediated by insulin-like



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growth factor or by GH itself is not known. Based on these mechanisms, recommendations were made that in GH-deficient patients under GH therapy $FT_4$ should not be targeted at the high-normal range [115].

## Conclusions and Perspectives

The real prevalence of CCH is higher than previously thought, probably around 1 in 15,000 neonates. The risk of children with severe forms of CCH for developing psychomotor delays is not to be underestimated. Diagnosis of CCH requires a high degree of suspicion at outpatient clinics. When inappropriately low serum TSH concentrations are associated with subnormal $FT_4$ levels, hypothyroidism of central origin should be investigated using an array of diagnostic tools at hand including dynamic stimulatory tests, imaging studies of the hypothalamic-pituitary area, determination of nocturnal thyrotropin surge or the use of indirect (biochemical, echocardiographic) indexes of the peripheral actions of thyroid hormones. Correlation of $FT_4$ and TSH levels through logarithmic or linear formulas may be useful in generating the suspicion for CCH. Idiopathic short stature may be the presenting clinical picture of mild forms of CCH.

In selected, well-characterized patients, nonroutine laboratory assays may demonstrate a reduced TSH bioactivity in serum samples of patients with CCH. Genetic testing of mutations or deletions in *TRHR*, *TSHB* and I*GSF1* genes definitively characterizes the clinical entity in front of the clinician. Further identification of novel clinical entities within the scope of CCH will hopefully lead to the possibility of unraveling novel human genes involved in the disease through dysregulation of known or yet unknown molecular mechanisms necessary for terminal differentiation of thyrotropes or modulating important hypothalamic-pituitary signaling pathways.

Levothyroxine is a safe and adequate treatment for CCH. As mentioned before, TRH preparations were administered in the past to patients with hypothalamic hypothyroidism, resulting in recovery of TSH biopotency [117]. While novel agonists of TRH like taltirelin became recently available, advantages and therapeutic applicability to CCH patients are currently not contemplated [118]. As for other pituitary defects, the definitive cure for CCH may come in the future from yet ongoing experimental approaches towards the in vitro generation of fully functional pituitary cell lineages to replace failing thyrotropic function [119, 120].

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